TITLE

HIGH GROWTH METHANOTROPHIC BACTERIAL STRAIN

This application claims the benefit of U.S. Provisional Application No. 60/229,858 filed September 1, 2000.

FIELD OF THE INVENTION

The invention relates to the field of microbiology. More specifically, the invention relates to the use of a novel methanotrophic bacterial strain capable of utilizing a central carbon pathway for more efficient production of commercially useful products.

BACKGROUND OF THE INVENTION

Methanotrophic bacteria are defined by their ability to use methane as their sole source of carbon and energy. Although methanol is an obligate intermediate in the oxidation of methane, the ability to grow on methanol alone is highly variable among the obligate methanotrophs due to its toxicity (Green, Peter. Taxonomy of Methylotrophic Bacteria. In: Methane and Methanol Utilizers (Biotechnology Handbooks 5) J. Colin Murrell and Howard Dalton eds. 1992 Pleanum Press NY, pp. 23-84). Methane monooxygenase is the enzyme required for the primary step in methane activation and the product of this reaction is methanol (Murrell et al., *Arch. Microbiol.* (2000), 173(5-6), 325-332). This reaction occurs at ambient temperatures and pressures, whereas chemical transformation of methane to methanol requires temperatures of hundreds of degrees and high pressure (Grigoryan, E. A., *Kinet. Catal.* (1999), 40(3), 350-363; WO 2000007718; US 5750821). It is this ability to transform methane under ambient conditions along with the abundance of methane that makes the biotransformation of methane a potentially unique and valuable process.

The commercial applications of biotransformation of methane have historically fallen broadly into three categories: 1) Production of single cell protein, (Sharpe D. H. BioProtein Manufacture (1989). Ellis Horwood series in applied science and industrial technology. New York: Halstead Press) (Villadsen, John, *Recent Trends Chem. React. Eng.*, [Proc. Int. Chem. React. Eng. Conf.], 2nd (1987), Volume 2, 320-33. Editor(s): Kulkarni, B. D.; Mashelkar, R. A.; Sharma, M. M. Publisher: Wiley East., New Delhi, India; Naguib, M.,Proc. OAPEC Symp. Petroprotein, [Pap.] (1980), Meeting Date 1979, 253-77 Publisher: Organ. Arab Pet. Exporting Countries, Kuwait, Kuwait); 2) epoxidation of alkenes for production of chemicals (U.S. 4,348,476); and 3) biodegradation of chlorinated pollutants

(Tsien et al., *Gas, Oil, Coal, Environ. Biotechnol.* 2, [Pap. Int. IGT Symp. *Gas, Oil, Coal, Environ. Biotechnol.*], 2nd (1990), 83-104. Editor(s): Akin, Cavit; Smith, Jared. Publisher: Inst. Gas Technol., Chicago, IL.;WO 9,633,821; Merkley et al., *Biorem. Recalcitrant Org.*, [Pap. Int. *In Situ* On-Site Bioreclam. Symp.], 3rd (1995), 165-74. Editor(s): Hinchee, Robert E; Anderson, Daniel B.; Hoeppel, Ronald E. Publisher: Battelle Press, Columbus, OH.: Meyer et al., *Microb. Releases* (1993), 2(1), 11-22). Only epoxidation of alkenes has experienced little commercial success due to low product yields, toxicity of products and the large amount of cell mass required to generate products.

Large-scale protein production from methane, termed single cell protein or SCP has been technically feasible and commercialized at large scale (Villadsen *supra*). However, SCP has been less than economically successful due to the relatively high cost to produce microbial protein compared to agriculturally derived protein (i.e. soy protein). Single cell protein is a relatively low value product and therefore economic production cannot tolerate heavy bioprocessing costs. For this reason the yield of the methanotrophic strain may be critical to the overall economic viability of the process. Microbial biomass produced by methanotrophic bacteria is typically very high in protein content (~70-80% by weight), which can restrict the direct use of this protein to certain types of animal feed.

The conversion of C1 compounds to complex molecules with C-C bonds is a difficult and capital intensive process by traditional chemical synthetic routes. Traditionally, methane is first converted to synthesis gas (mixtures of hydrogen, carbon monoxide and carbon dioxide), which is then used to produce other small molecular weight industrial precursors. Typically these are "commodity" type chemicals such as acetate, formaldehyde, or methanol. The basic problem is activation of the methane molecule which is thermodynamically very difficult to achieve by chemical means. "Activation" refers to the process of making the chemically unreactive methane molecule more reactive.

Methanotrophic bacteria contain enzymes (methane monooxygenases) which are capable of methane activation at ambient temperatures and pressures. Methane activation consists of oxygen insertion into methane to form methanol which is much more readily metabolized to more complex molecules within the cell. Two types of methane monooxygenase are found in methanotrophic bacteria. A

particulate methane monooxygenase (pMMO) has a narrow substrate specificity and is incapable of oxygen insertion into more complex molecules. Some, but not all methanotrophs may also contain a soluble methane monooxygenase (sMMO). This enzyme has been the subject of much investigation and proprietary claims due to its ability to oxygenate, or functionalize, a wide variety of aliphatic and aromatic molecules. This characteristic has been utilized for co-metabolic production processes where methanotrophs are fed both methane and a more complex molecule to be transformed by the sMMO. Numerous examples are reported of processes requiring both methane and, typically, a petroleum-derived feedstock such as toluene, naphthalene, or decane, where sMMO plays a role. However, the art is silent with respect to using methanotrophs for net synthesis of chemicals from methane as opposed to these co-metabolic transformations. For net synthesis, only inexpensive methane is required along with the ability to genetically engineer the strain to produce the desired chemical.

Methanotrophic cells can further build the oxidation products of methane (i.e. methanol and formaldehyde) into more complex molecules such as protein, carbohydrate and lipids. For example, under certain conditions methanotrophs are known to produce exopolysaccharides (Ivanova et al., *Mikrobiologiya* (1988), 57(4), 600-5; Kilbane, John J., II Gas, *Oil, Coal, Environ. Biotechnol.* 3, [Pap. IGT's Int. Symp.], 3rd (1991), Meeting Date 1990, 207-26. Editor(s): Akin, Cavit; Smith, Jared. Publisher: IGT, Chicago, IL). Similarly, methanotrophs are known to accumulate both isoprenoid compounds and carotenoid pigments of various carbon lengths (Urakami et al., *J. Gen. Appl. Microbiol.* (1986), 32(4), 317-41). Although these compounds have been identified in methanotrophs, they have not been microbial platforms of choice for production as these organisms have very poorly developed genetic systems, thereby limiting metabolic engineering for chemicals.

A necessary prerequisite to metabolic engineering of methanotrophs is a full understanding, and optimization, of the carbon metabolism for maximum growth and/or product yield. Obligate methanotrophs are typically thought to channel carbon from methane to useful products and energy via the Entner-Douderoff Pathway which utilizes the keto-deoxy phosphogluconate aldolase enzyme (Dijkhuizen, L., P.R. Levering, G.E. DeVries 1992. In: Methane and Methanol Utilizers (Biotechnology

Handbooks 5) J. Colin Murrell and Howard Dalton eds. 1992 Pleanum Press NY pp 149-181). This pathway is not energy-yielding as is the case for the Embden-Meyerhof pathway. Thus, utilization of the Entner-Douderoff pathway results in lower cellular production yields and a greater proportion of the carbon produced as carbon dioxide compared to organisms that use the Embden-Meyerhof pathway. Therefore, a more energy efficient carbon processing pathway would greatly enhance the commercial viability of a methanotrophic platform for the generation of materials.

As noted above, methanotrophic bacteria possess the potential to be commercially effective production platforms for materials such as single cell protein, exopolysaccharides, and long chain carbon molecules such as isoprenoids and carotenoid pigments. The usefulness of methanotrophs for production of a larger range of chemicals is constrained however, by several limitations including, relatively slow growth rates of methanotrophs, limited ability to tolerate methanol as an alternative substrate to methane, difficulty in genetic engineering, poor understanding of the roles of multiple carbon assimilation pathways present in methanotrophs, and potentially high costs due to the oxygen demand of fully saturated substrates such as methane. The problem to be solved therefore is to develop a fast-growing, high yielding methanotroph capable of receiving foreign genes via standard genetic procedures. Full and rapid resolution of central carbon pathways is essential for enabling pathway engineering and carbon flux management for new products.

Applicants have solved the stated problem by providing a methanotrophic bacterial strain capable of efficiently using either methanol or methane as a carbon substrate. The strain is also metabolically versatile in that it contains multiple pathways for the incorporation of carbon from formaldehyde into 3-C units. The discovery of a phosphofructokinase and fructose 1,6 bisphosphate aldolase in this strain suggests that it can utilize the more energetically favorable Embden-Meyerhof pathway in addition to the Entner-Douderoff pathways. The present strain is shown to be useful for the production of a variety of materials beyond single cell protein to include carbohydrates, pigments, terpenoid compounds and aromatic compounds. The formation of large amounts of carbohydrates from methane or methanol can be carried out by this strain. This is surprising and also enables this strain to be used for the production of

typical carbohydrate or sugar fermentation end-products such as alcohols, acids and ketones. The present strain was also shown to be capable of genetic exchange with donor species such as *Escherichia coli* via a standard genetic procedure known as bacterial conjugation. In this way, the strain can be engineered for net synthesis from methane to produce new classes of products other than those naturally produced.

SUMMARY OF THE INVENTION

The present invention provides a methanotrophic bacterial strain capable of growth on a C1 carbon substrate. The instant bacterial strain may be further characterized by the ability to grow rapidly and efficiently on either methanol or methane as a sole carbon source. This efficiency is due to the presence of a pyrophosphate linked phosphofructokinase enzyme within an operative Embden-Meyerhof pathway. This is a novel observation for methanotrophic bacteria. Functionally, the utilization of the Emben-Meyerhof pathway and pyrophosphate, instead of the Entner-Douderoff pathway reaction results in highly favorable cellular energetics which is manifested in higher yields, carbon conversion efficiency and growth rate.

The present strain also contains an enzyme system capable of reducing nitrate or nitrite with formation of gaseous nitrogen oxides. This capability is useful for reducing oxygen demand as well as for removing nitrates and nitrites in methane-containing environments such as landfills, wastewater treatment systems or anywhere that methane, oxygen and nitrates are present.

The ability to form large amounts of carbohydrates in the form of starch, polyglucose and/or extracellular polysaccharide is also useful for the production of carbohydrate-based products. Additionally *Methylomonas* 16a is only capable of growth on methane or methanol and is incapable of proliferating in the human body and thus is completely harmless and non-pathogenic. These characteristics make the strain ideally useful for the production of a wide range of products including animal feeds comprising variable carbohydrate/protein ratios.

The strain is shown to be capable of genetic exchange and expression of foreign genes. Additionally the present strain may be identified by the characteristic 16sRNA sequence as set forth in SEQ ID NO:81.

Additionally the present invention provides methods for the production of single cell protein, carbohydrates, and carotenoid pigments, or higher value mixtures of protein, pigments and carbohydrates. Additionally the strain may be used as a denitrifying agent for the

conversion of nitrate or nitrite to nitrous oxide with methane or methanol as carbon source.

Accordingly the invention provides a high growth methanotrophic bacterial strain which:

(a) grows on a C1 carbon substrate selected from the group consisting of methane and methanol; and

(b) comprises a functional Embden-Meyerhof carbon pathway, said pathway comprising a gene encoding a pyrophosphate dependent phosphofructokinase enzyme, the gene selected from the group consisting of:

(a) an isolated nucleic acid molecule encoding the amino acid sequence as set forth in SEQ ID NO:6;

(b) an isolated nucleic acid molecule that hybridizes with (a) under the following hybridization conditions: 0.1X SSC, 0.1% SDS, 65°C and washed with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS;

(c) an isolated nucleic acid molecule comprising a first nucleotide sequence encoding a polypeptide of at least 437 amino acids that has at least 63% identity based on the Smith-Waterman method of alignment when compared to a polypeptide having the sequence as set forth in SEQ ID NO:6; and

(d) an isolated nucleic acid molecule that is complementary to (a), (b) or (c).

Optionally the present strain may comprise at least one gene encoding a fructose bisphosphate aldolase enzyme as part of the functional Embden-Meyerhof carbon pathway. Additionally, the present strain may optionally contain a functional Entner-Douderoff carbon pathway, where the Entner-Douderoff carbon pathway comprises at least one gene encoding a keto-deoxy phosphogluconate aldolase.

In one embodiment the present strain may optionally contain other carbon flux genes encoding polypeptides selected from the group consisting of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18 and 20.

In another embodiment the present strain may possess a denitrification pathway where the pathway may optionally comprise genes encoding polypeptides having the amino acid sequences selected from the

group consisting of SEQ ID NO:40, 42, 44, 46, 48, 50, 52, 54, 56, 58 and 60.

In another embodiment the present strain may contain a set of exopolysaccharide synthesizing enzymes where the exopolysaccharide synthesizing enzymes may have the amino acid sequences selected from the group consisting of SEQ ID NO:22, 24, 26, 28, 30, 32, 34, 36, and 38.

In a more specific embodiment the present strain may comprise genes encoding isoprenoid synthesizing enzymes where the enzymes are selected from the group consisting of SEQ ID NO:62, 64, 66, 68, 70, 72, 74, 86, and 78.

In a preferred embodiment the invention provides a method for the production of single cell protein comprising:

a) contacting the present high growth methanotrophic bacterial strain with a C1 carbon substrate, selected from the group consisting of methane and methanol, in a suitable medium for a time sufficient to permit the expression and accumulation of single cell protein; and

b) optionally recovering the single cell protein.

It is an additional object of the invention to provide a method for the biotransformation of a nitrogen containing compound selected from the group consisting of ammonia, nitrate, nitrite, and dinitrogen comprising, contacting the present high growth methanotrophic bacterial strain with a C1 carbon substrate selected from the group consisting of methane or methanol, in the presence of the nitrogen containing compound, in a suitable medium for a time sufficient to permit the biotransformation of the nitrogen containing compound.

Similarly it is an object of the present invention to provide a method for the production of a feed product comprising protein, carbohydrates and pigment comprising the steps of:

a) contacting the high growth methanotrophic bacterial strain of the present invention with a C1 carbon substrate in a suitable medium for a time sufficient to permit the expression and accumulation of the feed product; and

b) optionally recovering the feed product.

Optionally the relative compositions of protein, carbohydrate and pigment are altered through the up-regulation or down-regulation of any one of the genes encoding the proteins selected from the group consisting

of SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, and 69.

In a preferred embodiment the invention provides a method of identifying a high growth methanotrophic bacterial strain comprising:

(a) growing a sample suspected of containing a high growth methanotrophic bacterial strain on a suitable growth medium in the presence of methane as a sole carbon source;

(b) identifying colonies that grow under the conditions of step (a);

(c) analyzing the colonies identified in step (b) for the presence of pyrophosphate dependent phosphofructokinase activity.

BRIEF DESCRIPTION OF THE DRAWINGS, SEQUENCE DESCRIPTIONS AND BIOLOGICAL DEPOSITS

Figure 1 shows the growth of *Methylomonas* 16a compared to the growth of *Methylococcus capsulatus* under identical growth conditions.

Figure 2 is a plot of optical density vs. methanol concentration for a culture of *Methylomonas* 16a grown on methanol alone.

Figure 3 represents a schematic of the Entner-Douderoff and Embden-Meyerhof pathways in Methylomonas 16a showing microarray expression results numerically ranked in order of decreasing expression level.

Figure 4 shows oxygen uptake by a cell suspension of *Methylomonas* 16a, in arbitrary units to detect oxygen consumption.

Figure 5 shows oxygen uptake by a cell suspension of *Methylomonas* 16a, in arbitrary units to detect oxygen consumption before and after sodium nitrite was injected into the incubation.

Figure 6 is a plot of the concentration of O_2 and N_2O evolved per hour vs. the concentration of O_2 in the medium of a cell suspension of *Methylomonas* 16a under aerobic conditions.

The invention can be more fully understood from the following detailed description and the accompanying sequence descriptions which form a part of this application.

The following sequence descriptions and sequences listings attached hereto comply with the rules governing nucleotide and/or amino acid sequence disclosures in patent applications as set forth in 37 C.F.R. §1.821-1.825. The Sequence Descriptions contain the one letter code for nucleotide sequence characters and the three letter codes for amino acids as defined in conformity with the IUPAC-IYUB standards

described in *Nucleic Acids Research 13*:3021-3030 (1985) and in the *Biochemical Journal 219 (No. 2)*:345-373 (1984) which are herein incorporated by reference. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

Description	SEQ ID Nucleic acid	SEQ ID Peptide
Phosphoglucomutase: carbon Flux	1	2
Glucose 6 phosphate isomerase:Carbon flux	3	4
Phosphofructokinase pyrophosphate dependent: Carbon Flux	5	6
6-Phosphogluconate dehydratase:Carbon flux	7	8
Glucose 6 phosphate 1 dehydrogenase:Carbon Flux	9	10
Transaldolase: Carbon Flux	11	12
Transaldolase: Carbon Flux	13	14
Fructose bisphosphate aldoslase:Carbon Flux	15	16
Fructose bisphosphate aldoslase:Carbon Flux	17	18
KHG/KDPG Aldolase :Carbon Flux	19	20
ugp: Exopolysaccharaide	21	22
*gumD:*Exopolysaccharaide	23	24
wza:Exopolysaccharaide	25	26
*epsB:*Exopolysaccharaide	27	28
*epsM:*Exopolysaccharaide	30	20
*waaE:*Exopolysaccharaide	31	32
*epsV:*Exopolysaccharaide	33	34
*gumH:*Exopolysaccharaide	35	36
glycosyl transferase:Exopolysaccharaide	37	38
nirF: Denitrification	39	40
nirD: Denitrification	41	42
nirL:Denitrification	43	44
nirG:Denitrification	45	46
nirH:Denitrification	47	48
nirJ:Denitrification	49	50
nasA:Denitrification	51	52
norC:Denitrification	53	54
norB:Denitrification	55	56
norZ:Denitrification	57	58
norS:Denitrification	59	60
*dxs:*Terpenoid synthesis	61	62
dxr: Terpenoid synthesis	63	64

Description	SEQ ID Nucleic acid	SEQ ID Peptide
ispF: Terpenoid synthesis	65	66
ispD: Terpenoid synthesis	67	68
pyrG: Terpenoid synthesis	69	70
lspA: Terpenoid synthesis	71	72
lspE: Terpenoid synthesis	73	74
crtN: Terpenoid synthesis	75	76
crtN1: Terpenoid synthesis	77	78
Particulate monooxygenase	79	80
16sRNA for Methylomonas 16a	81	-

Applicants made the following biological deposits under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure:

Depositor Identification Reference	International Depository Designation	Date of Deposit
Methylomonas 16a	ATCC PTA 2402	August 21 2000

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes the isolation and characterization of a high growth methanotrophic bacterial strain useful for the production of biomass including proteins, carbohydrates and pigments. The present strain is typed by 16sRNA as a *Methylomonas sp.* and is referred to herein as *Methylomonas 16a*. In addition, the strain may be useful for the production of mixtures of proteins, carbohydrates and pigments for the purpose of generating animal feeds. The strain possesses the advantage of an active Embden-Meyerhof carbon flux pathway having a pyrophosphate dependent phosphofructokinase gene, which conveys certain energetic advantages to the strain as a production platform for various materials and biomass. Additionally the strain naturally possesses an active isoprenoid pathway for the generation of pigments indigenous to the strain. In this disclosure, a number of terms and abbreviations are used. The following definitions are provided.

The term "Embden-Meyerhof pathway" refers to the series of biochemical reactions for conversion of hexoses such as glucose and fructose to important cellular 3 carbon intermediates such as glyceraldehyde-3-phosphate, dihydroxyacetone phosphate, phosphophenol pyruvate and pyruvate. These reactions typically proceed with net yield of biochemically useful energy in the form of ATP. The key enzymes unique to the Embden-Meyerhof pathway are phosphofructokinase and fructose-1,6 bisphosphate aldolase.

The term "Entner-Douderoff pathway" refers to a series of biochemical reactions for conversion of hexoses such as glucose or fructose to important 3 carbon cellular intermediates such as pyruvate and glyceraldehyde-3-phosphate without any net production of biochemically useful energy. The key enzymes unique to the Entner-Douderoff pathway are the 6 phosphogluconate dehydratase and the ketodeoxyphosphogluconate aldolase.

The term "diagnostic" as it relates to the presence of a gene in a pathway means where a gene having that activity is identified, it is evidence of the presence of that pathway. Within the context of the present invention the presence of a gene encoding a pyrophosphate dependant phosphofructokinase is "diagnostic" for the presence of the Embden-Meyerhof carbon pathway and the presence of gene encoding a ketodeoxyphosphogluconate aldolase is "diagnostic" for the presence of the Entner-Douderoff carbon pathway.

The term "Yield" is defined herein as the amount of cell mass produced per gram of carbon substrate metabolized.

The term "carbon conversion efficiency" is a measure of how much carbon is assimilated into cell mass and is calculated assuming a biomass composition of $CH_2O_{0.5}N_{0.25}$.

The term "high growth methanotrophic bacterial strain" refers to a bacterium capable of growth with methane or methanol as a sole carbon and energy source and which possesses a functional Embden-Meyerhof carbon flux pathway resulting in a yield of cell mass per gram of C1 substrate metabolized. The specific "high growth methanotrophic bacterial strain" described herein is referred to as "*Methylomonas* 16a" or "16a", which terms are used interchangeably.

The term "a C1 carbon substrate" refers to any carbon-containing molecule that lacks a carbon-carbon bond. Examples are methane,

methanol, formaldehyde, formic acid, methylated amines, and methylated thiols.

The term "functional denitrifying enzymatic pathway" refers to a series of enzymes which sequentially reduce nitrate or nitrite to more reduced products such as nitric oxide, nitrous oxide or ultimately dinitrogen. This process may or may not be energy yielding.

The term "denitrification" refers to the process of converting nitrates or nitrites to gaseous dinitrogen or other gaseous nitrogen oxides. To facilitate denitrification the present strain comprises genes encoding a number of enzymes in the denitrification pathway including: the *nir* genes (*nirD, nirF, nirG, nirH, nirJ, nirL* and *nirS*) encoding the nitrite reductase which catalyzes the reduction of nitrite (NO_2) to nitric oxide, the *nasA* gene, encoding nitrate reductase which catalyzes the reduction of nitrate (NO_3) to nitrite (NO_2); and the *nor* genes (*norB, norC* or *norZ*) encoding a nitric oxide reductase which catalyzes the reduction of nitric oxide (NO) to nitrous oxide (N_2O).

The term "isoprenoid compound" refers to any compound which is derived via the pathway beginning with isopentenyl pyrophosphate and formed by the head to tail condensation of isoprene units which may be of 5, 10, 15, 20, 30 or 40 carbons in length. The term "isoprenoid pigment" refers to a class of compounds which typically have strong light absorbing properties and are derived from the head to tail condensation of 5, 10, 15, 20, 25, 30 or 40 carbon isoprene chains. These isoprene chains are ultimately derived from isopentenyl pyrophosphate. A number of genes and gene products are associated with the present strain encoding the isoprenoid biosynthetic pathway including the *dxs* gene, encoding 1-deoxyxylulose-5-phosphate synthase, the *dxr* gene, encoding 1-deoxyxylulose-5-phosphate reductoisomerase, the *"ispD,"* gene encoding the 2C-methyl-D-erythritol cytidyltransferase enzyme, the *"ispE"* gene encoding the 4-diphosphocytidyl-2-C-methylerythritol kinase, the *"ispF"* gene encoding a 2C-methyl-d-erythritol 2,4-cyclodiphosphate synthase, the *"pyrG"* gene, encoding a CTP synthase, the *"ispA"* gene, encoding geranyltransferase or farneseyl diphosphate synthase and the *"ctrN"* and "*ctrN* 1" genes, encoding diapophytoene dehydrogenase.

The term "single cell protein" will be abbreviated "SCP" and refers to a protein derived from organisms that exist in the unicellular, or single cell,

state. This includes unicellular bacteria, yeasts, fungi or eukaryotic single cell organisms such as algae.

The term "extracellular polysaccharide" or "exocellular polysaccharide" will be abbreviated "ESP" and refers to a polysaccharide produced by methanotrophic bacteria typically comprising a carbohydrate "backbone" polymer as cross-linking carbohydrate polymers. These polymers are excreted on the outside of the microbial cell and may function in adhesion to surfaces or as a response to environmental stress. The present strain comprises a number of genes encoding various steps in the synthesis of extracellular polysaccharide including the *"ugp"* gene encoding UDP-glucose pyrophosphorylase, the *"gumD"* and *"waaE"* genes encoding glycosyltransferases, the *"wza"* and *"epsB"* genes, encoding polysaccharide export proteins, the *"epsM"* gene, encoding a polysaccharide biosynthesis related protein, and the *"epsV"* gene, encoding a sugar transferase.

The term "carbohydrate" refers to any sugar containing constituent, particularly storage forms, such as glycogen or starch and extracellular polysaccharides.

The term "fermentation product" refers to products derived from the fermentation of any carbohydrate formed by the methanotrophic bacterium from methane or methanol.

The term "particulate methane monooxygenase" will be abbreviated as "pMMO" and will refer to a membrane-associated methane monooxygenase which inserts oxygen in to the enzyme substrate.

The terms "soluble methane monooxygenase" will be abbreviated as "sMMO" and will refer a to soluble or cytoplasmic methane monooxygenase - localized in the cytoplasm.

As used herein, an "isolated nucleic acid fragment" is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a

native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

The term "percent identity", as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in: Computational Molecular Biology (Lesk, A. M., ed.) Oxford University Press, NY (1988); Biocomputing: Informatics and Genome Projects (Smith, D. W., ed.) Academic Press, NY (1993); Computer Analysis of Sequence Data, Part I (Griffin, A. M., and Griffin, H. G., eds.) Humana Press, NJ (1994); Sequence Analysis in Molecular Biology (von Heinje, G., ed.) Academic Press (1987); and Sequence Analysis Primer (Gribskov, M. and Devereux, J., eds.) Stockton Press, NY (1991). Preferred methods to determine identity are designed to give the best match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Sequence alignments and percent identity calculations may be performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS. 5*:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5.

Suitable nucleic acid fragments (isolated polynucleotides of the present invention) encode polypeptides that are at least about 70% identical, preferably at least about 80% identical to the amino acid sequences reported herein. Preferred nucleic acid fragments encode amino acid sequences that are about 85% identical to the amino acid sequences reported herein. More preferred nucleic acid fragments encode amino acid sequences that are at least about 90% identical to the amino acid sequences reported herein. Most preferred are nucleic acid fragments that encode amino acid sequences that are at least about 95% identical to the amino acid sequences reported herein. Suitable nucleic acid fragments not only have the above homologies but typically encode a polypeptide having at least 50 amino acids, preferably at least 100 amino acids, more preferably at least 150 amino acids, still more preferably at least 200 amino acids, and most preferably at least 250 amino acids.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength. Hybridization and washing conditions are well known and exemplified in Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989), particularly Chapter 11 and Table 11.1 therein (entirely incorporated herein by reference). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Stringency conditions can be adjusted to screen for moderately similar fragments, such as homologous sequences from distantly related organisms, to highly similar fragments, such as genes that duplicate functional enzymes from closely related organisms. Post-hybridization washes determine stringency conditions. One set of preferred conditions uses a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 min, then repeated with 2X SSC, 0.5% SDS at 45°C for 30 min, and then repeated twice with 0.2X SSC, 0.5% SDS at 50°C for 30 min. A more preferred set of stringent conditions uses higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2X SSC, 0.5% SDS was increased to 60°C. Another preferred set of highly stringent conditions uses two final washes in 0.1X SSC, 0.1% SDS

at 65°C. An additional preferred set of stringent conditions include 0.1X SSC, 0.1% SDS, 65°C and washed with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS).

Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T*m* for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T*m*) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T*m* have been derived (see Sambrook et al., *supra*, 9.50-9.51). For hybridizations with shorter nucleic acids, i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook et al., *supra*, 11.7-11.8). In one embodiment the length for a hybridizable nucleic acid is at least about 10 nucleotides. Preferable a minimum length for a hybridizable nucleic acid is at least about 15 nucleotides; more preferably at least about 20 nucleotides; and most preferably the length is at least 30 nucleotides. Furthermore, the skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as length of the probe.

The term "sequence analysis software" refers to any computer algorithm or software program that is useful for the analysis of nucleotide or amino acid sequences. "Sequence analysis software" may be commercially available or independently developed. Typical sequence analysis software will include but is not limited to the GCG suite of programs (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI), BLASTP, BLASTN, BLASTX (Altschul et al., *J. Mol. Biol.* 215:403-410 (1990), and DNASTAR (DNASTAR, Inc. 1228 S. Park St. Madison, WI 53715 USA), and the FASTA program incorporating the Smith-Waterman algorithm (W. R. Pearson, *Comput. Methods Genome Res.,* [Proc. Int. Symp.] (1994), Meeting Date 1992, 111-20. Editor(s): Suhai, Sandor. Publisher: Plenum, New York, NY). Within the context of

this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters which originally load with the software when first initialized.

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989) (hereinafter "Maniatis"); and by Silhavy, T. J., Bennan, M. L. and Enquist, L. W., Experiments with Gene Fusions, Cold Spring Harbor Laboratory Cold Press Spring Harbor, NY (1984); and by Ausubel, F. M. et al., Current Protocols in Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience (1987).

The present invention provides a unique methanotrophic bacterial strain, useful for the production of a variety materials from C1 carbon sources such as methane and methanol. The strain is referred to herein as *Methylomonas* 16a, and is characterized by rapid doubling time, high yield and the presence of genes encoding both the Entner-Douderoff carbon pathway as well as the Embden-Meyerhof pathway, allowing for versatility in carbon flux management and higher efficiency of carbon incorporation. The strain has been shown to produce a variety of food and feed products such as single cell protein, exopolysaccharide and starch. The strain has particularly high value in the production of food and feed materials as it is possible to manipulate the various concentrations of protein, carbohydrate and starch all within the same organism. This capability will permit strains to be uniquely tailored for individual specific food and feed applications. Additionally the strain has demonstrated utility in the production of terpenoid and carotenoid compounds, useful as pigments and as monomers in polymeric materials.

Isolation of *Methylomonas* 16a

The original environmental sample containing *Methylomonas* 16a was obtained from pond sediment. The pond sediment was inoculated directly into a defined mineral medium under 25% methane in air. Methane was used as the sole source of carbon and energy. Growth was followed until the optical density at 660 nm was stable, whereupon the culture was transferred to fresh medium such that a 1:100 dilution was achieved. After

3 successive transfers with methane as the sole carbon and energy source the culture was plated onto defined minimal medium agar and incubated under 25% methane in air. Many methanotrophic bacterial species were isolated in this manner. However, *Methylomonas* 16a was selected as the organism to study due to the rapid growth of colonies, large colony size, its ability to grow on minimal media, and pink pigmentation indicative of an active biosynthetic pathway for carotenoids.

Methanotrophs are classified into three metabolic groups ("Type I", "Type X" or "Type II") based on the mode of carbon incorporation, morphology, %GC content and the presence or absence of key specific enzymes. Example 4, Table 2 shows key traits determined for *Methylomonas* 16a in relation to the three major groupings of methanotrophs. The strain clearly falls into the Type I grouping based on every trait, with the exception of nitrogen fixation. It is generally well accepted that these organisms do not fix nitrogen. Therefore, *Methylomonas* 16a appears unique in this aspect of nitrogen metabolism.

16SrRNA extracted from the strain was sequenced and compared to known 16SrRNAs from other microorganisms. The data showed 96% identity to sequences from *Methylomonas sp.* KSP III and *Methylomonas sp.* strain LW13. Based on this evidence, as well as the other physiological traits described in Table 2 (Example 4), it was concluded that the strain was a member of the genus *Methylomonas*.

Metabolic and Physiological Characterization of *Methylomonas 16a*

Carbon Metabolism: The present methanotrophic bacterial strain, *Methylomonas* 16a, converts methane to methanol via a methane monooxygenase as the first step in carbon utilization. The methane monooxygenase present in the strain is a particulate, as opposed to a soluble, monooxygenase. Particulate methane monooxygenases (pMMO) are well known in the art (Murrell et al., *Arch. Microbiol.* (2000), 173(5-6), 325-332) and many have been isolated and sequenced. pMMO's are characterized by their narrow substrate specificity as opposed to sMMO's which are less discriminating. For this reason the pMMO enzyme is favored for the production of bulk chemicals since the sMMO is likely to modify many of the chemical intermediates needed for the efficient production of a specific product.

The gene and gene product corresponding to the pMMO isolated from the present strain have been sequenced and functionally identified on

the basis of homology comparisons to sequences in publicly available databases. The instant sequence is highly homologous to that isolated from *Methylococcus capsulatus* (GenBank B57266).

The present strain contains several anomalies in the carbon utilization pathway. For example, based on genome sequence data, the strain is shown to contain genes for two pathways of hexose metabolism. The Entner-Douderoff Pathway utilizing the keto-deoxy phosphogluconate aldolase enzyme is present in the strain. Is generally well accepted that this is the operative pathway in obligate methanotrophs. Also present, however, is the Embden-Meyerhof pathway which utilizes the fructose bisphosphate aldolase enzyme. It is well known that this pathway is either not present or not operative in obligate methanotrophs. Energetically, the latter pathway is most favorable and allows greater yield of biologically useful energy, ultimately resulting in greater yield production of cell mass and other cell mass-dependent products in *Methylomonas* 16a. The activity of this pathway in the present 16a strain has been confirmed through microarray data and biochemical evidence measuring the reduction of ATP. Although the 16a strain has been shown to possess both the Embden-Meyerhof and the Entner-Douderoff pathway enzymes the data suggests that the Embden-Meyerhof pathway enzymes are more strongly expressed than the Entner-Douderoff pathway enzymes. This result is surprising and counter to existing beliefs concerning the glycolytic metabolism of methanotrophic bacteria. Applicants have discovered other methanotrophic bacteria having this characteristic, including for example, *Methylomonas clara* and *Methylosinus sporium.* It is likely that this activity has remained undiscovered in methanotrophs due to the lack of activity of the enzyme with ATP, the typical phosphoryl donor for the enzyme in most bacterial systems.

A particularly novel and useful feature of the Embden-Meyerhof pathway in strain 16a is that the key phosphofructokinase step is pyrophosphate dependent instead of ATP dependent. This feature adds to the energy yield of the pathway by using pyrophosphate instead of ATP (Example 6). Because of its significance in providing an energetic advantage to the strain, this gene in the carbon flux pathway is considered diagnostic for the present strain.

Comparison of the pyrophosphate dependent phosphofructokinase gene sequence (SEQ ID NO:5) and deduced amino acid sequence (SEQ

ID NO:6) to public databases reveals that the most similar known sequences is about 63% identical to the amino acid sequence reported herein over a length of 437 amino acid using a Smith-Waterman alignment algorithm (W. R. Pearson, *Comput. Methods Genome Res.,* [Proc. Int. Symp.] (1994), Meeting Date 1992, 111-20. Editor(s): Suhai, Sandor. Publisher: Plenum, New York, NY). More preferred amino acid fragments are at least about 80%-90% identical to the sequences herein. Most preferred are nucleic acid fragments that are at least 95% identical to the amino acid fragments reported herein. Similarly, preferred pyrophosphate dependent phosphofructokinase encoding nucleic acid sequences corresponding to the instant gene are those encoding active proteins and which are at least 80% identical to the nucleic acid sequences of reported herein. More preferred pyrophosphate dependent phosphofructokinase nucleic acid fragments are at least 90% identical to the sequences herein. Most preferred are pyrophosphate dependent phosphofructokinase nucleic acid fragments that are at least 95% identical to the nucleic acid fragments reported herein.

Accordingly the invention provides a high growth methanotrophic bacterial strain which:

(a) grows on a C1 carbon substrate selected from the group consisting of methane and methanol; and

(b) comprises a functional Embden-Meyerhof carbon pathway, said pathway comprising a gene encoding a pyrophosphate dependent phosphofructokinase enzyme, the gene selected from the group consisting of:

(a) an isolated nucleic acid molecule encoding the amino acid sequence as set forth in SEQ ID NO:6;

(b) an isolated nucleic acid molecule that hybridizes with (a) under the following hybridization conditions: 0.1X SSC, 0.1% SDS, 65°C and washed with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS;

(c) an isolated nucleic acid molecule comprising a first nucleotide sequence encoding a polypeptide of at least 437 amino acids that has at least 63% identity based on the Smith-Waterman method of alignment when compared to a polypeptide having the sequence as set forth in SEQ ID NO:6; and

(d) an isolated nucleic acid molecule that is complementary to (a), (b) or (c).

Methane and methanol are the only substrates shown to support growth of *Methylomonas* 16a. The strain is grown on defined medium without the addition of complex growth factors. Methanol utilization is reported to typically require "adaptation" and growth on methanol concentration ranging from 0.1% to 3% is also reported as "variable". *Methylomonas* 16a was shown to grow on methanol concentrations as high as 600 mM (2.4%) without adaptation and with good yield. (Figure 2).

In methanotrophic bacteria methane is converted to biomolecules via a cyclic set of reactions known as the ribulose monophosphate pathway or RuMP cycle. This pathway is comprised of three phases, each phase being a series of enzymatic steps. The first step is "fixation" or incorporation of C-1 (formaldehyde) into a pentose to form a hexose or six carbon sugar. This occurs via a condensation reaction between a 5 carbon sugar (pentose) and formaldehyde and is catalyzed by hexulose monophosphate synthase. The second phase is termed "cleavage" and results in splitting of that hexose into two 3 carbon molecules. One of those three carbon molecules is recycled back through the RuMP pathway and the other 3 carbon fragment is utilized for cell growth. In methanotrophs and methylotrophs the RuMP pathway may occur as one of three variants. However, only two of these variants are commonly found: the FBP/TA (fructose bisphosphotase/Transaldolase) or the KDPG/TA (keto deoxy phosphogluconate/transaldolase) pathway. (Dijkhuizen L., G.E. Devries. The physiology and biochemistry of aerobic methanol-utilizing gram negative and gram positive bacteria. In: Methane and Methanol Utilizers 1992, ed. Colin Murrell and Howard Dalton. Plenum Press, NY).

The present strain is unique in the way it handles the "cleavage" steps as genes were found that carry out this conversion via fructose bisphosphate as a key intermediate. The genes for fructose bisphosphate aldolase and transaldolase were found clustered together on one piece of DNA. Secondly the genes for the other variant involving the keto deoxy phosphogluconate intermediate were also found clustered together. Available literature teaches that these organisms (methylotrophs and methanotrophs) rely solely on the KDPG pathway and that the FBP-dependent fixation pathway is utilized by facultative methylotrophs

(Dijkhuizen et al., *supra*). Therefore the latter observation is expected, whereas the former is not. The finding of the FBP genes in an obligate methane utilizing bacterium is both surprising and suggestive of utility. The FBP pathway is energetically favorable to the host microorganism due to the fact that less energy (ATP) is utilized than is utilized in the KDPG pathway. Thus organisms that utilize the FBP pathway may have an energetic advantage and growth advantage over those that utilize the KDPG pathway. This advantage may also be useful for energy-requiring production pathways in the strain. By using this pathway, a methane-utilizing bacterium may have an advantage over other methane utilizing organisms as production platforms for either single cell protein or for any other product derived from the flow of carbon through the RuMP pathway.

Accordingly the present invention provides a *Methylomonas* having two distinct carbon flux pathways, comprising genes and gene products as set forth in SEQ ID NO:1-20, and encoding both a pyrophosphate dependent phosphofructokinase pyrophosphate and a keto-deoxy phosphogluconate (KDPG) aldolase. Comparison of the KDPG aldolase gene sequence (SEQ ID NO:19) and deduced amino acid sequence (SEQ ID NO:20) to public databases reveals that the most similar known sequences is about 59% identical to the amino acid sequence of reported herein over a length of 212 amino acid using a Smith-Waterman alignment algorithm (W. R. Pearson, *supra).* More preferred amino acid fragments are at least about 80%-90% identical to the sequences herein. Most preferred are nucleic acid fragments that are at least 95% identical to the amino acid fragments reported herein. Similarly, preferred KDPG aldolase encoding nucleic acid sequences corresponding to the instant gene are those encoding active proteins and which are at least 80% identical to the nucleic acid sequences of reported herein. More preferred KDPG aldolase nucleic acid fragments are at least 90% identical to the sequences herein. Most preferred are KDPG aldolase nucleic acid fragments that are at least 95% identical to the nucleic acid fragments reported herein.

It is thus an object of the invention to provide a high growth methanotrophic bacterial strain having the ability to grow exclusively on either methane or methanol, comprising a functional Embden-Meyerhof carbon pathway, said pathway comprising a gene encoding a pyrophosphate dependent phosphofructokinase enzyme and at least one

gene encoding a keto-deoxy phosphogluconate aldolase enzyme, selected from the group consisting of:

(a) an isolated nucleic acid molecule encoding the amino acid sequence as set forth in SEQ ID NO:20;

(b) an isolated nucleic acid molecule that hybridizes with (a) under the following hybridization conditions: 0.1X SSC, 0.1% SDS, 65°C and washed with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS;

(c) an isolated nucleic acid molecule comprising a first nucleotide sequence encoding a polypeptide of at least 212 amino acids that has at least 59% identity based on the Smith-Waterman method of alignment when compared to a polypeptide having the sequence as set forth in SEQ ID NO:20; and

(d) an isolated nucleic acid molecule that is complementary to (a), (b) or (c).

In addition to the pyrophosphate dependent phosphofructokinase enzyme and keto-deoxy phosphogluconate aldolase enzyme, the strain comprises other carbon flux genes including an FBP aldolase, phosphoglucomutase, pyrophosphate dependent phosphofructokinase pyrophosphate, 6-Phosphogluconate dehydratase, and a glucose-6 phosphate-1 dehydrogenase. The phosphoglucomutase is responsible for the interconversion of glucose-6-phosphate to glucose-1-phosphate, which feeds into either the Entner-Douderoff or Embden-Meyerhof carbon flux pathways. As shown in Figure 3, fructose-6-phosphate may be convert to either glucose-6-phosphase by glucose phosphate isomerase (Entner-Douderoff) or to fructose-1,6-bisphosphate (FBP) by a phosphofructokinase (Embden-Meyerhof). Following the Embden-Meyerhof pathway, FBP is then taken to two three-carbon moieties (dihydroxyacetone and 3-phosphoglyceraldehyde) by the FBP aldolase. Returning to the Entner-Douderoff system, glucose-6-phosphate is taken to 6-phosphogluconate by a glucose-6-phosphate dehydrogenase which is subsequently taken to 2-keto-3-deoxy-6-phosphogluconate (KDPG) by a 6 phosphogluconate dehydratase. The KDPG is then converted to two three-carbon moieties (pyruvate and 3-phosphoglyceraldehyde) by a KDPG aldolase. Thus the Embden-Meyerhof and Entner-Douderoff pathways are rejoined at the level of 3-phosphoglyceraldehyde.

Identification of High Growth Methanotrophic Bacteria

Although the present 16a strain has been isolated fortuitously, it is contemplated that the present teaching will enable the general identification and isolation of similar strains. For example, the key characteristics of the present high growth strain are that it is an obligate methanotroph, using only either methane or methanol as a sole carbon source; and it possesses a functional Embden-Meyerhof pathway, and particularly a gene encoding a pyrophosphate dependent phosphofructokinase. Methods for the isolation of methanotrophs are common and well known in the art (See for example Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA., or Deshpande, Mukund V., *Appl. Biochem. Biotechnol.*, 36, 227, (1992)). Similarly pyrophosphate dependent phosphofructokinase has been well characterized in mammalian systems and assay methods have been well developed (see for example Schliselfeld et al. *Clin. Biochem.* (1996), 29(1), 79-83; Clark et al., *J. Mol. Cell. Cardiol.* (1980), 12(10), 1053-64). The contemporary microbiologist will be able to use these techniques to identify the present high growth strain.

The specific strain of the present invention possesses a specific pyrophosphate dependent phosphofructokinase having the amino acid sequence as set forth in SEQ ID NO:6. The present strain may be further characterized by analyzing a methanotrophic bacterial strain for the presence of the gene encoding this enzyme.

It is therefore an object of the invention to provide a method of identifying a high growth methanotrophic bacterial strain comprising:

(a) growing a sample suspected of containing a high growth methanotrophic bacterial strain on a suitable growth medium in the presence of methane as a sole carbon source;

(b) identifying colonies that grow on the conditions of step (a);

(c) analyzing the colonies identified in step (b) for the presence of pyrophosphate dependent phosphofructokinase activity.

Growth Characteristics: The presence of the above mentioned carbon flux characteristics was previously unknown in methanotrophic bacteria and may explain the rapid growth rate and the increased carbon conversion efficiency of this strains and other strains possessing this pathway, relative to strain that do not have this pathway. The present *Methylomonas* 16a has been shown to grow on methane with a doubling time of only 2.5 h. This is a very high growth rate

and is an obvious advantage for commercial use as well as for the genetic manipulations performed in development of the strain. Additionally, *Methylomonas* has no requirement for organic growth factors such as yeast extract or other costly fermentation additives. The strain requires only methane or methanol, inorganic minerals, oxygen and water for optimum growth, giving the present strain an advantage for large scale growth at low cost.

Particularly noteworthy is the high yield of the present strain. Yield is defined herein as the amount of cell mass produced per gram of carbon substrate metabolized. The present strain has shown the ability to produce greater than 0.8 and preferably greater than 1.0 grams of cell mass per gram of methane metabolized. Similarly the present strain has shown the ability to produce greater than 0.30 and preferably greater than 0.45, more preferably greater than 0.5 cell mass per gram of methanol metabolized.

Carbon conversion efficiency is another measure of how much carbon is assimilated into cell mass. Carbon conversion efficiency is expressed in units of g/mol methane (1 g dry wt/g methane) / g/ mol biomass. Carbon conversion efficiency is calculated assuming a biomass composition of $CH_2O_{0.5}N_{0.25}$. The present strain will have a particularly high carbon conversion efficiency where an efficiency of greater than 40 is common, an efficiency of greater than 50 is preferred, a conversion of greater than 65 is highly preferred and an efficient of greater than 70 is most preferred.

Methanol Utilization: *Methylomonas* 16a is shown to grow at methanol concentrations as high as 600 mM . Typically methanol can be toxic at these concentrations to some methanotrophic bacteria. *Methylomonas* 16a can tolerate up to about 2.4% methanol which is at the upper end of the known spectrum of methanol tolerance for methanotrophic bacteria (Green, Peter, Taxonomy of Methylotrophic Bacteria. In: Methane and Methanol Utilizers (Biotechnology Handbooks 5) J. Colin Murrell and Howard Dalton eds., 1992 Pleanum Press NY, pp 23-84). This feature again allows for much lower capital costs in reactor design since tolerance for methanol is higher necessitating reactors with fewer mixing ports (i.e. lower construction costs). This issue (high reactor costs due to mixing requirements to overcome methanol toxicity) is a major drawback to growth of methanotrophic bacteria on methanol.

Glycogen Production: *Methylomonas* 16a has been shown to produce in excess of 50% of its weight as glycogen during active growth on methanol and significant amounts of glycogen during active (non-stress associated) growth on methane. This aspect is useful for the production of mixtures of protein and

carbohydrate to serve a wider array of animal feed nutritional needs as compared to other obligate methanotrophs producing only protein as the sole product. Alternatively, this trait enables *Methylomonas* 16a to serve as a host strain for the production of glycogen from methane or methanol. Furthermore, internal hexose metabolism is clearly occurring in *Methylomonas* 16a. Thus the organism can serve as host for the production of chemical products typically considered to be only produced by carbohydrate metabolism. Accordingly the invention provides a *Methylomonas* strain having the ability to produce in excess of 50% of its weight of glycogen when grown on methanol, where about 20% to about 40% is typical.

Pigment and Terpenoid Production: The present *Methylomonas* strain is useful for the production of a variety of pigments and particularly the isoprenoid pigments. This class of pigments are known to have strong light absorbing properties and are derived from the head to tail condensation of 5, 10, 15, 20, 25, 30 or 40 carbon isoprene chains. One specific pigment identified in the present strain is a C-30 carotenoid. The content of this pigment is very high in the cell and is indicative of naturally high carbon flow through the isoprenoid pathway. This aspect provides the basis for viewing the isoprenoid pathway as a "backbone production pathway" for isoprenoid-derived products. It is contemplated for example that high value carotenoids such as astaxanthin, β-carotene, canthaxanthin, and lutein may be produced by the instant organism.

Additionally the present strain is expected to have the ability to produce various isoprenoid compounds. Isoprenoids are an extremely large and diverse group of natural products that have a common biosynthetic origin based on a single metabolic precursor known as isopentenyl diphosphate (IPP). The group of natural products known as isoprenoids includes all substances that are derived biosynthetically from the 5-carbon compound isopentenyl diphosphate. Isoprenoid compounds are also referred to as "terpenes" or "terpenoids", which is the term used in the designation of the various classes of these examples (Spurgeon and Porter, Biosynthesis of Isoprenoid Compounds, pp 3-46, A Wiley-Interscience Publication (1981)). Isoprenoids are ubiquitous compounds found in all living organisms. Some of the well-known examples of isoprenoids are steroids (triterpenes), carotenoids (tetraterpenes), and squalene, just to name a few.

The biosynthesis of such compounds typically involve the enzyme isopentenyl pyrophosphate and are formed by the head to tail condensation of isoprene units which may be of 5, 10, 15, 20, 30 or 40 carbons in length.

It is contemplated that other, related, small cyclic molecules such as limonene, menthol and geraniol may be produced in the present strain via the introduction of the appropriate plant-derived terpene synthases. Thus the isoprenoid pathway may be viewed as a platform pathway for production of complex cyclic and unsaturated molecules from methane or methanol. This capability is unique to biology, purely chemical processes cannot convert C-1 compounds to cyclic molecules with any degree of specificity.

Many steps in isoprenoid pathways are known. For example, the initial steps of the alternate pathway involve the condensation of 3-carbon molecules (pyruvate and C1 aldehyde group, D-glyceraldehyde 3-Phosphate), to yield a 5-carbon compound (D-1-deoxyxylulose-5-phosphate). Lois et al. has reported a gene, *dxs*, that encodes D-1-deoxyxylulose-5-phosphate synthase (DXS) that catalyzes the synthesis of D-1-deoxyxylulose-5-phosphate in *E. coli* (*Proc. Natl. Acad. Sci. USA* 95: 2105-2110 (1998)).

Next, the intramolecular rearrangement of D-1-deoxyxylulose-5-phosphate occurs by an unspecified reduction process for the formation of 2-C-methyl-D-erythritol-4-phosphate. One of the enzymes involved in the reduction process is D-1-deoxyxylulose-5-phosphate reductoisomerase (DXR). Takahashi et al. reported the *dxr* gene product catalyzes the formation of 2-C-methyl-D-erythritol-4-phosphate in the alternate pathway in *E. coli* (*Proc. Natl. Acad. Sci. USA* 95: 9879-9884 (1998)).

Steps converting 2-C-methyl-D-erythritol-4-phosphate to isopentenyl monophosphate are not well characterized although some steps are known. 2-C-methyl-D-erythritol-4-phosphate is converted into 4-diphosphocytidyl-2C-methyl-D-erythritol in a cytosine triphosphate (CTP) dependent reaction by the enzyme encoded by non-annotated gene *ygbP*, encoding a 2C-methyl-d-erythritol cytidylyltransferase. Rondich et al. reported a YgbP protein in *E. coli* that catalyzes the reaction mentioned above (*Proc. Natl. Acad. Sci. USA* 96:11758-11763 (1999)). Recently, *ygbP* gene was renamed as *ispD* as a part of the *isp* gene cluster. The 2 position hydroxy group of 4-diphosphocytidyl-2C-methyl-D-erythritol can

be phosphorylated in an ATP dependent reaction by a 4-diphosphocytidyl-2-C-methylerythritol kinase encoded by the *ychB* gene. Luttgen et al. has reported a YchB protein in *E. coli* that phosphorylates 4-diphosphocytidyl-2C-methyl-D-erythritol, resulting in 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate (*Proc. Natl. Acad. Sci. USA* 97:1062-1067 (2000)). Recently, the *ychB* gene was renamed as *ispE* as a part of the *isp* gene cluster.

Herz et al. reported that the *ygbB* gene product (2C-methyl-d-erythritol 2,4-cyclodiphosphate synthase) in *E. coli* converts 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate to 2C-methyl-D-erythritol 2,4-cyclodiphosphate in a CTP dependent reaction. 2C-methyl-D-erythritol 2,4-cyclodiphosphate can be further converted into carotenoids through the carotenoid biosynthesis pathway (*Proc. Natl. Acad. Sci. USA* 97:2486-2490 (2000)). Recently, the *ygbB* gene was renamed as *ispF* as a part of *isp* gene cluster.

Both reactions catalyzed by the YgbB and YgbP enzymes are carried out in CTP dependent manner. Thus CTP synthase plays an important role in the isoprenoid pathway. PyrG encoded by the *pyrG* gene in *E. coli* was determined to encode CTP synthase (Weng et al., *J. Biol. Chem.*, 261:5568-5574 (1986)).

Following several reactions not yet characterized, isopentenyl monophosphate is formed. Isopentenyl monophosphate is converted to an isopentenyl diphosphate (IPP) by isopentenyl monophosphate kinase enzyme encoded by the *ipk* gene (Lange and Croteau, *Proc. Natl. Acad. Sci. USA* 96:13714-13719 (1999)).

Prenyltransferases constitute a broad group of enzymes catalyzing the consecutive condensation of isopentenyl diphosphate (IPP), resulting in the formation of prenyl diphosphates of various chain lengths. Homologous genes of prenyl transferase have highly conserved regions in their amino acid sequences. Ohto et al. reported three prenyl transferase genes in cyanobacterium *Synechococcus elongatus* (*Plant Mol. Biol.* 40:307-321 (1999)). They are geranylgeranyl (C20) diphosphate synthase, farnesyl (C15) diphosphate synthase (*ispA*), and another prenyltransferase that can catalyze the synthesis of five prenyl diphosphates of various length.

Further down in the isoprenoid biosynthesis pathway, more genes are involved in the synthesis of specific isoprenoids. As an example, the

crtN gene that was found in *Heliobacillus mobilis* (*Proc. Natl. Acad. Sci. USA* 95:14851-14856 (1998)) encodes a diapophytoene dehydrogenase that is a part of the carotenoid biosynthesis pathway.

Although some of the genes involved in isoprenoid pathways are well known, the presence of genes involved in the isoprenoid pathway of *Methylomonas sp.* is rare. It is surprising therefore to find all of the above mentioned genes in the present strain (SEQ ID NO:61-SEQ ID NO:78). Tgus suggests that the present strain will be useful for the production of a variety of terpenoids. Accordingly the invention provides a *Methylomonas* strain having the genes and gene products as set forth in SEQ ID NO:61-SEQ ID NO:78, encoding a D-1-deoxyxylulose-5-phosphate synthase, a D-1-deoxyxylulose-5-phosphate reductoisomerase, 2C-methyl-d-erythritol 2,4-cyclodiphosphate synthase, a 2C-methyl-d-erythritol cytidylyltransferase, a CTP synthase, a Geranyltranstransferase (also farnesyl-diphosphate synthase), a 4-diphosphocytidyl-2-C-methylerythritol kinase, and a diapophytoene dehydrogenase.

Production of Single Cell Protein: The present strain is useful for the production of single cell protein (SCP) which has value in the food and feed industries. Methods for the use of methanotrophs as production platforms for the production of SCP are well known in the art (see for example US 4,795,708; Shojaosadati et al., *Amirkabir* (1996), 8(30), 33-41). The present strain is well suited for this application due to its advantages in carbon flux and reduced oxygen consumption in the presence of a nitrogen source. The strain is well suited for the production of single cell protein under either aerobic or anaerobic conditions.

The present strain compares favorably with other known strains, producing up to about 1.3 g protein/dry weight/ g methane and up to about 0.45 g protein/dry weight/ g methanol.

Production of exopolysaccharides: Polysaccharides are sugar polymers that have been used widely as a thickener in food and non-food industries (Sanford et al. *Pure & Appl. Chem.* 56: 879-892 (1984); Sutherland, *Trends Biotechnol*, 16(1): 41-6 (1998)). They can be found in food products such as salad dressing, jam, frozen food, bakery products, canned food and dry food. Many other applications include suspending agents for pesticides, paints and other coating agents. They can act as flocculent, binders, film-formers, lubricants and friction reducers.

Furthermore, exopolysaccharides are commonly used in the oil field for oil recovery.

Traditionally, industrially useful polysaccharides have been derived from algal and plant sources. Over the past decade polysaccharides derived from microbes have been found increased usage (Sanford et al. *Pure & Appl. Chem.* 56: 879-892 (1984); Sutherland, *Trends Biotechnol*, 16(1): 41-6 (1998)).

Many other genes involved in exopolysaccharide biosynthesis have been characterized or sequenced from other organisms. The *epsB* gene encodes the *EpsB* protein that is probably involved in polymerization and/or export of *EPS*, and has been sequenced in *Ralstonia sola* (Huang et al, *Mol. Microbiol.* 16: 977-989 (1995)). The *espM* gene encoding the *EspM* protein has been found in the *esp* gene cluster from *Streptococcus thermophilus* (Stingele et al, *J. Bacteiol.* 178: 1680-1690 (1996)). Another putative polysaccharide export protein, WZA, is identified in *E. coli.* (Blattner et al., *Science* 277: 1453-1474 (1997)). Finally, the *epsV* gene encodes the *EpsV* protein, a transferase which transfers the sugar to polysaccharide intermediates, and it has also been sequenced in *Streptococcus thermophilus* (Bourgoin et al., *Plasmid* 40: 44-49 (1998); Bourgoin,F., et al., *Gene* 233:151-161 (1999)).

In spite of the abundance of information regarding genes encoding microbial exopolysaccharides, no genes involved in this pathway have been isolated or characterized from C1 utilizing organisms, such as *Methylomonas*. As noted above, microbial exopolysaccharides have a variety of uses and it would be an advantage to synthesize this material from an abundant and inexpensive carbon source such as methane.

Surprisingly, the present *Methylomonas* 16a has been shown to produce extrapolysaccharides at high levels. The genes encoding the relevant polysaccharide synthesis pathways have been isolated and characterized and are described along with their gene products in SEQ ID NO:21-SEQ ID NO:38.

Accordingly, the present invention provides a *Methylomonas* strain having the ability to synthesize exopolysaccharides and having genes encoding the *ugp, gumD,* wza, *epsB, epsM, waaE, epsV, gumH* and glycosyl transferase proteins associated with microbial polysaccharide biosynthesis.

Denitrification: The presence of denitrification enzymes in obligate methanotrophs is unknown. The present strain contains a pathway comprised of genes and gene products as set forth in SEQ ID NO:39-SEQ ID NO:60. A novel feature of the present *Methylomonas 16a* is the ability to utilize a nitrogen source at low oxygen tensions as an additional "electron sink" for reducing equivalents derived from methane or methanol. Nitrogen sources may include, but are not limited to, nitrite, nitrate, ammonium and dinitrogen. The strain is shown to reduce nitrate or nitrite to nitrous oxide which is a gaseous end-product. The utility in this process is that nitrate is very soluble as well as inexpensive and use of nitrate mitigates against the high energy requirement for maintaining dissolved oxygen in the process. In fact, nitrate is utilized as an accessory oxidant in some waste water treatment systems (Koch, Gerhard; Siegrist, Hansruedi Verbandsber. - Verb. Schweiz. Abwasser- Gewaesserschutzfachleute (1998), 522 (Optimierungsmassnahmen bei Stark Belasteten Belebungsanlagen), 33-48).

In non-methanotrophic denitrifiers, the microbial process known as denitrification is catalyzed by a series of enzymes which together reductively convert nitrate to gaseous dinitrogen. The steps and intermediates in the process as shown below, together with the enzyme names and gene designations define the scope of the process under consideration.

1. $NO_3 \rightarrow NO_2$ Respiratory nitrate reductase (*Nar* genes).
2. $NO_2 \rightarrow NO$ Respiratory nitrite reductase (*Nir* genes)
3. $NO \rightarrow N_2O$ Nitric oxide reductase (*Nor* genes)
4. $N_2O \rightarrow N_2$ Nitrous oxide reductase (*Nos* genes)

Ecologically, the result of these processes is removal of nitrogen from soils (denitrification). However, nitrate can also be viewed as a supplemental or alternative oxidant to oxygen. This is due to the very positive redox potential of the denitrification process.

A second major microbial process is referred to as nitrification and that is comprised of the following set of reactions, enzymes and genes.

1. NH_4 → NH_2OH Ammonia monooxygenase (*amo* genes)
2. NH_2OH → NO_2 (Hydroxylamine oxidoreductase)
3. NO_2 → NO_3 (Nitrite oxidase)

Nitrification is an oxidative process generating nitrate in soils whereas denitrification is a reductive process depleting nitrate in soils.

It is well known that obligatory methanotrophic bacteria belong to the group of nitrifying bacteria. This is due to the ability of methane monooxygenase which is found in all obligate methanotrophs to oxygenate ammonia to form hydroxylamine in a reaction identical to that of ammonia monooxygenase and analogous to methane oxygenation to form methanol. The hydroxylamine is then further metabolized enzymatically to nitrite. Nitrite oxidation to nitrate can occur enzymatically or spontaneously in air via chemical oxidation. However methanotrophic bacteria have been indirectly associated with denitrification by virtue of their association with denitrifying bacteria such as *Hyphomicrobium* species (Amaral, J.A. Archambault, C. S.R. Richards, R. Knowles 1995. *FEMS Microbiology Ecology* 18 289-298). The respiratory processes described above are distinct from the reduction of nitrate or nitrite for cellular assimilation. The former respiratory process is energy yielding whereas the latter assimilatory process provides nitrogen for incorporation into cellular mass. The assimilatory process relies upon pyridine nucleotide linked nitrate or nitrite reductases. These enzymes are widely found in nature including the methanotrophic bacteria. Growth of methanotrophs on nitrate as a sole nitrogen source for biosynthesis is well known in the existing literature (Hanson R.S. A.I. Netrusov, K. Tsuji. 1992. The obligate methanotrophic bacteria Methylococcus, Methylomonas, and Methylosinus. In: The Prokaryotes 2nd ed. Ch 18. Pp 2350-2363, A. Balows, H.G. Truper, M. Dworkin, W. Harder, K-H Schleifer eds. Springer Verlag).

The functionality of the genes described herein (SEQ ID NO:39-SEQ ID NO:60) lie in the respiratory reduction of nitrate or nitrite to gaseous N_2O. All genes required to perform this function have been shown to be present in *Methylomonas* 16a both by sequence analysis and physiological reduction of nitrogen containing compounds. Additionally the genes encoding enzymes necessary for the biotransformation of ammonia (nitrification) are also present.

The advantages to the presence of this denitrification capability in an obligate methanotroph are at least two fold:

1. Nitrate may replace or supplement oxygen as an electron acceptor needed for growth. This can be advantageous for large scale cost-effective cultivation with highly reduced feedstocks that require excessive oxygen demand leading to excessive costs for mass-transfer of gaseous oxygen into solution.
2. Methanotrophic denitrification may be used to remove soluble nitrates from waters or processes where nitrates or other oxygenated nitrogen derivatives are problematic.

Due to the ability of *Methylomonas* 16a to convert ammonia to nitrite combined with the ability to convert nitrite to nitrous oxide demonstrated in the present invention, *Methylomonas* 16a and other methanotrophs which efficiently reduce nitrite can be used as agents to remove ammonia from process waters, waste waters, or natural waters or agricultural effluents for the purpose of clean up and detoxification

Gene Transfer into Methylomonas 16a : *Methylomonas* 16a has been shown to accept and express genes form other organisms including *Escherichia coli* and yeast. Several plasmid vectors have been identified which facilitate both gene transfer from a donor organism and expression of the gene in *Methylomonas* 16a. Thus the strain can be genetically engineered.

Production of Food and Feed Substrates

It will be appreciated that the present *Methylomonas* 16a strain has the ability to produce, not only proteins, polysaccharides and pigments individually, but may also be engineered to produce a uniquely tailored food or feed product comprising specific quantities and desirable mixtures of these materials. This characteristic of the present strain has significant commercial value.

For example, different livestock animal types may have different nutritional requirements in terms of the relative proportions of protein to carbohydrate. Many carnivorous aquatic fish species, for example, have very high protein requirements. Ruminant livestock ,on the other hand, thrive on higher fiber/carbohydrate diets. *Methylomonas* 16a has the capacity to form large amounts of carbohydrate, under certain conditions, in addition to the cellular protein which is always produced. Genes

involved in gluconeogenesis (glycogen formation) or glycogen degradation might be altered or regulated such that glycogen content could either be decreased or increased. Thus the composition of the crude cell mass could be modulated to target high protein feed markets (lower carbohydrate) or alternatively, higher carbohydrate lower protein feed markets. The ability to engineer the composition of the microbe precludes the need to artificially formulate protein/carbohydrate ratios by exogenous additions.

Carotenoid pigments play a role in terms of providing coloration for many aquatic fish and crustacean species as well as providing antioxidant benefit. (Nelis H.J., De Leenheer 1991. *J. Appl. Bacteriol.* 70:181-191). *Methylomonas* 16a, unlike many commercially utilized methanotrophs (i.e. *Methylococcus capsulatus*) has a natural carotenoid pigment production pathway which produces high levels of a pink pigment that is similar, but not structurally identical, with such high value carotenoids as astaxanthin. Modification of this pathway by addition of genes involved in the final steps of astaxanthin synthesis or other high value carotenoids will result in the ability of this strain to produce these carotenoids. In this way *Methylomonas* 16a will be uniquely useful as an animal feed production strain in which the ratios of protein/carbohydrate/pigments may be tailored to suit particular nutritional needs. In this way, Methylomonas may be utilized as a way to deliver higher value components to other sources of plant protein or carbohydrate and thus circumvent the problem of genetic engineering of these plants for the higher value traits.

Methods of manipulating genetic pathways are common and well known in the art. Selected genes in a particular pathway may be upregulated or down regulated by variety of methods. Additionally, competing pathways in the organism may be eliminated or sublimated by gene disruption and similar techniques.

Once a key genetic pathway has been identified and sequenced specific genes may be upregulated to increase the output of the pathway. For example, additionally copies of the targeted genes may be introduced into the host cell on multicopy plasmids such as pBR322. Alternatively, the target genes may be modified so as to be under the control of non-native promoters. Where it is desired that a pathway operate at a particular point in a cell cycle or during a fermentation run, regulated or inducible promoters may used to replace the native promoter of the target gene.

Similarly, in some cases the native or endogenous promoter may be modified to increase gene expression. For example, endogenous promoters can be altered *in vivo* by mutation, deletion, and/or substitution (see, Kmiec, U.S. Patent 5,565,350; Zarling et al., PCT/US93/03868).

Alternatively it may be necessary to reduce or eliminate the expression of certain genes in the target pathway or in competing pathways that may serve as competing sinks for energy or carbon. Methods of down-regulating genes for this purpose have been explored. Where sequence of the gene to be disrupted is known, one of the most effective methods of gene down regulation is targeted gene disruption where foreign DNA is inserted into a structural gene so as to disrupt transcription. This can be effected by the creation of genetic cassettes comprising the DNA to be inserted (often a genetic marker) flanked by sequence having a high degree of homology to a portion of the gene to be disrupted. Introduction of the cassette into the host cell results in insertion of the foreign DNA into the structural gene via the native DNA replication mechanisms of the cell. (See for example Hamilton et al. (1989) *J. Bacteriol.* 171:4617-4622; Balbas et al. (1993) *Gene* 136:211-213; Gueldener et al. (1996) *Nucleic Acids Res.* 24:2519-2524; and Smith et al. (1996) *Methods Mol. Cell. Biol.* 5:270-277.)

Antisense technology is another method of down regulating genes where the sequence of the target gene is known. To accomplish this, a nucleic acid segment from the desired gene is cloned and operably linked to a promoter such that the anti-sense strand of RNA will be transcribed. This construct is then introduced into the host cell and the antisense strand of RNA is produced. Antisense RNA inhibits gene expression by preventing the accumulation of mRNA which encodes the protein of interest. The person skilled in the art will know that special considerations are associated with the use of antisense technologies in order to reduce expression of particular genes. For example, the proper level of expression of antisense genes may require the use of different chimeric genes utilizing different regulatory elements known to the skilled artisan.

Although targeted gene disruption and antisense technology offer effective means of down regulating genes where the sequence is known, other less specific methodologies have been developed that are not sequence based. For example, cells may be exposed to a UV radiation and then screened for the desired phenotype. Mutagenesis with chemical

agents is also effective for generating mutants and commonly used substances include chemicals that affect nonreplicating DNA such as HNO_2 and NH_2OH, as well as agents that affect replicating DNA such as acridine dyes, notable for causing frameshift mutations. Specific methods for creating mutants using radiation or chemical agents are well documented in the art. See for example Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA., or Deshpande, Mukund V., *Appl. Biochem. Biotechnol.*, 36, 227, (1992).

Another non-specific method of gene disruption is the use of transposoable elements or transposons. Transposons are genetic elements that insert randomly in DNA but can be latter retrieved on the basis of sequence to determine where the insertion has occurred. Both *in vivo* and *in vitro* transposition methods are known. Both methods involve the use of a transposable element in combination with a transposase enzyme. When the transposable element or transposon, is contacted with a nucleic acid fragment in the presence of the transposase, the transposable element will randomly insert into the nucleic acid fragment. The technique is useful for random mutageneis and for gene isolation, since the disrupted gene may be identified on the basis of the sequence of the transposable element. Kits for *in vitro* transposition are commercially available (see for example The Primer Island Transposition Kit, available from Perkin Elmer Applied Biosystems, Branchburg, NJ, based upon the yeast Ty1 element; The Genome Priming System, available from New England Biolabs, Beverly, MA; based upon the bacterial transposon Tn7; and the EZ::TN Transposon Insertion Systems, available from Epicentre Technologies, Madison, WI, based upon the Tn5 bacterial transposable element.

Within the context of the present invention it may be useful to modulate the expression of the identified biosynthetic pathways. For example, it has been noted that the present *Methylomonas* 16a comprises genes encoding both the Entner-Douderoff and Embden-Meyerhof carbon flux pathways. Because the Embden-Meyerhof pathway is more energy efficient it may be desirable to over-express the genes in this pathway. Additionally, it is likely that the Entner-Douderoff pathway is a competitive pathway and inhibition of this pathway may lead to increased energy efficiency in the Embden-Meyerhof system. This might be accomplished

by selectively using the above described methods of gene down regulation on the sequence encoding the keto-deoxy phosphogluconate aldolase (SEQ ID NO:9) or any of the other members of the Entner-Douderoff system and upregulating the gene encoding the fructose bisphosphatase aldolase of the Embden-Meyerhof system (SEQ ID NO:5 OR 7). In this fashion the carbon flux in the present *Methylomonas* 16a may be optimized. Additionally, where the present strain has been engineered to produce specific organic materials such as aromatics for monomer production, optimization of the carbon flux pathway will lead to increased yields of these materials.

In a similar fashion the genes encoding the key enzymes involved in isoprenoid or pigment synthesis may be modulated. For example, the present invention provides a number of genes encoding key enzymes in the terpenoid pathway leading to the production of pigments and smaller isoprenoid compounds. The isolated genes include the *dxs* and *dsr* genes, the *ispA, D, E, F,* and *G* genes, the *pyrG* gene, and *crtN* genes. In particular it may be useful to up-regulate the initial condensation of 3-carbon molecules (pyruvate and C1 aldehyde group, D-glyceraldehyde 3-Phosphate), to yield the 5-carbon compound (D-1-deoxyxylulose-5-phosphate) mediated by the *dxs* gene. Alternatively, if it is desired to produce a specific non-pigmented isoprenoid, it may be desirable to disrupt various genes at the downstream end of the pathway. For example, it may be desirable to use gene disruption or antisense inhibition of the *crtN* gene (known to encode diapophytoene dehydrogenase) if a smaller, upstream terpenoid is the desired product of the pathway.

As has been noted, the present strain has the ability to product polysaccharides in large amounts. This process is governed by a set of genes including the *ugp* gene, *gumD* and *H* genes, the *epsB, M,* and *V* genes and the *waaD* gene. In this pathway it may be of particular importance to up-regulate the *espB* gene involved in polymerization and/or export of the polysaccharide, or the *epsV* gene which controls the transfer of sugar to polysaccharide intermediates.

In this fashion the present strain, or a similar strain may be engineered to produce specific compositions of materials or specific combinations of protein, polysaccharides and pigments for use as a food and feed product.

EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

GENERAL METHODS

Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, (1989) (Maniatis) and by T. J. Silhavy, M. L. Bennan, and L. W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and by Ausubel, F. M. et al., Current Protocols in Molecular Biology, pub. by Greene Publishing Assoc. and Wiley-Interscience (1987).

Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found as set out in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994)) or by Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition, Sinauer Associates, Inc., Sunderland, MA (1989). All reagents, restriction enzymes and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "mL" means milliliters, "L" means liters.

Microbial Cultivation and Preparation of Cell Suspensions, and associated analyses.

Methylomonas 16a is typically grown in serum stoppered Wheaton bottles using a gas/liquid ratio of at least 8:1 (i.e. 20 mL of Nitrate liquid media) media in a Wheaton bottle (Wheaton Scientific, Wheaton IL) of 160 mL total volume. The standard gas phase for cultivation contained 25% methane in air. These conditions comprise growth conditions and the cells are referred to as growing cells. In all cases the cultures were grown at 30°C with constant shaking in a Lab-Line rotary shaker unless otherwise specified.

Cells obtained for experimental purposes were allowed to grow to maximum optical density (O.D. 660 ~ 1.0). Harvested cells were obtained by centrifugation in a Sorval RC-5B centrifuge using a SS-34 rotor at 6000 rpm for 20 min. These cell pellets were resuspended in 50 mM HEPES buffer pH 7. These cell suspensions are referred to as washed, resting cells.

Microbial growth was assessed in all experiments by measuring the optical density of the culture at 660 nm in an Ultrospec 2000 UV/Vis spectrophotometer (Pharmacia Biotech, Cambridge England) using a 1 cm light path cuvet. Alternatively microbial growth was assessed by harvesting cells from the culture medium by centrifugation as described above and resuspending the cells in distilled water with a second centrifugation to remove medium salts. The washed cells were then dried at 105°C overnight in a drying oven for dry weight determination.

Methane concentration was determined as described by Emptage et al. (1997 *Env. Sci. Technol.* 31:732-734), hereby incorporated by reference.

Nitrate medium for *Methylomonas* 16A

Nitrate liquid medium, also referred to herein as "defined medium" was comprised of various salts mixed with solution 1 as indicated below or where specified the nitrate was replaced with 15 mM ammonium chloride.

Solution 1 Composition for 100 fold concentrated stock solution of trace minerals.

	MW	*Conc. (mM)*	*g per L*
Nitriloacetic acid	191.1	66.9	12.8
$CuCl_2 \times 2H_2O$	170.48	0.15	0.0254
$FeCl_2 \times 4H_2O$	198.81	1.5	0.3
$MnCl_2 \times 4H_2O$	197.91	0.5	0.1
$CoCl_2 \times 6H_2O$	237.9	1.31	0.312
$ZnCl_2$	136.29	0.73	0.1
H_3BO_3	61.83	0.16	0.01
$Na_2MoO_4 \times 2H_2O$	241.95	0.04	0.01
$NiCl_2 \times 6H_2O$	237.7	0.77	0.184

Mix the gram amounts designated above in 900 mL of H_2O, adjust to pH=7, and add H_2O to an end volume of 1 L. Keep refrigerated.

Nitrate liquid medium:

	MW	*Conc. (mM)*	*g per L*
$NaNO_3$	84.99	10	0.85
KH_2PO_4	136.09	3.67	0.5
Na_2SO_4	142.04	3.52	0.5
$MgCl_2 \times 6H_2O$	203.3	0.98	0.2
$CaCl_2 \times 2H_2O$	147.02	0.68	0.1
1 M HEPES (pH 7)	238.3		50 mL
Solution 1			10 mL

Dissolve in 900 mL H_2O. Adjust to pH=7, and add H_2O to give 1 L.

For agar plates: Add 15 g of agarose in 1 L of medium, autoclave, let cool down to 50°C, mix, and pour plates.

Nitrate and Nitrite Assays

1 mL samples of cell culture were taken and filtered through a 0.2 micron Acrodisc filter to remove cells. The filtrate from this step contains the nitrite or nitrate to be analyzed. The analysis was performed on a Dionex ion chromatograph 500 system (Dionex, Sunnyvale CA) with an AS3500 autosampler. The column used was a 4 mm Ion-Pac AS11-HC separation column with an AG-AC guard column and an ATC trap column. All columns are provided by Dionex.

The mobile phase was a potassium hydroxide gradient from 0 to 50 mM potassium hydroxide over a 12 min time interval. Cell temperature was 35°C with a flow rate of 1 mL/min.

Gene Isolation and Characterization

A number of genes encoding specific identifying enzymes were isolated and sequenced from *Methylomonas* 16a. These include distinguishing genes found in the Entner-Douderoff carbon flux pathway the Embden-Meyerhof carbon flux pathway, genes encoding a denitrification pathway, genes encoding an isoprenoid synthesis pathway, and genes encoding a pathway for the synthesis of exopolysaccharides. These genes were sequenced and functionally characterized by comparison of their respective sequences to information in public nucleic acid and protein databases according to the following procedures.

Genomic DNA was isolated from *Methylomonas* 16a according to standard protocols. Genomic DNA and library construction were prepared according to published protocols (Fraser et al The Minimal Gene Complement of *Mycoplasma genitalium*; *Science* 270, 1995). A cell pellet was resuspended in a solution containing 100 mM Na-EDTA pH 8.0, 10 mM tris-HCl pH 8.0, 400 mM NaCl, and 50 mM MgCl2.

Genomic DNA preparation After resuspension, the cells were gently lysed in 10% SDS, and incubated for 30 min at 55°C. After incubation at room temperature, proteinase K was added to 100 μg/mL and incubated at 37°C until the suspension was clear. DNA was extracted twice with tris-equilibrated phenol and twice with chloroform. DNA was precipitated in 70% ethanol and resuspended in a solution containing 10 mM tris-HCl and 1 mM Na-EDTA (TE) pH 7.5. The DNA solution was treated with a mix of RNAases, then extracted twice with tris-equilibrated phenol and twice with chloroform. This was followed by precipitation in ethanol and resuspension in TE.

Library construction 200 to 500 μg of chromosomal DNA was resuspended in a solution of 300 mM sodium acetate, 10 mM tris-HCl, 1 mM Na-EDTA, and 30% glycerol, and sheared at 12 psi for 60 sec in an Aeromist Downdraft Nebulizer chamber (IBI Medical products, Chicago, IL). The DNA was precipitated, resuspended and treated with Bal31 nuclease. After size fractionation, a fraction (2.0 kb, or 5.0 kb) was excised, cleaned and a two-step ligation procedure was used to produce a high titer library with greater than 99% single inserts.

Sequencing A shotgun sequencing strategy approach was adopted for the sequencing of the whole microbial genome (Fleischmann, Robert et al Whole-Genome Random sequencing and assembly of *Haemophilus influenzae* Rd *Science* , 269: 1995).

Sequence was generated on an ABI Automatic sequencer using dye terminator technology (US 5366860; EP 272007) using a combination of vector and insert-specific primers. Sequence editing was performed in either DNAStar (DNA Star Inc.,) or the Wisconsin GCG program (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI) and the CONSED package (version 7.0). All sequences represent coverage at least two times in both directions.

Microarray of gene expression

Amplification of DNA regions for the construction of DNA microarray: Specific primer pairs were used to amplify each protein specifying ORF of *Methylomonas* sp. strain 16a. Genomic DNA (10-30 ng) was used as the template. The PCR reactions were performed in the presence of HotStart Taq™ DNA polymerase (Qiagen, Valencia, CA) and the dNTPs (Gibco BRL Life Science Technologies, Gaithersberg, MD). Thirty-five cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and polymerization at 72°C for 2 min were conducted. The quality of PCR reactions was checked with electrophresis in a 1% argarose gel. The DNA samples were purified by the high-throughput PCR purification kit from Qiagen.

Arraying amplified ORFs. Before arraying, an equal volume of DMSO (10 µL) and DNA (10 µL) sample was mixed in 384-well microtiter plates. A generation II DNA spotter (Molecular Dynamics, Sunnyvale, CA) was used to array the samples onto coated glass slides (Telechem, Sunnyvale, CA). Each PCR product was arrayed in duplicate on each slide. After cross-linking by UV light, the slides were stored under vacuum in a desiccator at room temperature.

RNA isolation: *Methylomonas* 16a was cultured in a defined medium with ammonium or nitrate (10 mM) as nitrogen source under 25% methane in air. Samples of the minimal medium culture were harvested when the O.D. reaches 0.3 at A_{600} (exponential phase). Cell cultures were harvested quickly and ruptured in RLT buffer [Qiagen RNeasy Mini Kit, Valencia, CA] with a beads-beater (Bio101, Vista, CA). Debris was pelleted by centrifugation for 3 min at 14,000 x g at 4°C. RNA isolation

was completed using the protocol supplied with this kit. After on-column DNAase treatment, the RNA product was eluted with 50-100 μL RNAase-free. RNA preparations were stored frozen at either -20 or –80°C.

Synthesis of fluorescent cDNA from total RNA. RNA samples (7 to 15 μg) and random hexamer primers (6 μg; Gibco BRL Life Science Technologies) were diluted with RNAase-free water to a volume of 25 μL. The sample was denatured at 70°C for 10 min and then chilled on ice for 30 seconds. After adding 14 μL of labeling mixture, the annealing was accomplished by incubation at room temperature for 10 min. The labeling mixture contained 8 μL of 5x enzyme buffer, 4 μL DTT (0.1M), and 2 μL of 20x dye mixture. The dye mixture consisted of 2 mM of each dATP, dGTP, and dTTP, 1 mM dCTP, and 1 mM of Cy3-dCTP or Cy5-dCTP. After adding 1 to 1.5 μL of SuperScript II reverse transcriptase (200 units/mL, Life Technologies Inc., Gaithersburg, MD), cDNA synthesis was allowed to proceed at 42°C for 2 hr. The RNA was removed by adding 2 μL NaOH (2.5 N) to the reaction. After 10 min of incubation at 37°C, the pH was adjusted with 10 μL of HEPES (2M). The labeled cDNA was then purified with a PCR purification kit (Qiagen, Valencia, CA). Labeling efficiency was monitored using either A_{550} for Cy3 incorporation, or A_{650} for Cy5.

Fluorescent labeling of genomic DNA. Genomic DNA was nebulized to approximately 2 kb pair fragments. Genomic DNA (0.5 to 1 μg) was mixed with 6 μg of random hexamers primers (Gibco BRL Life Science Technologies) in 15 μL of water. The mix was denatured by put at boiling water for 5 minutes. Then anneal on ice for 30 sec before put at room temperature. Then 2 μL 5x Buffer 2 (Gibco BRL) and 2 ul dye mixture were added. The component of dye mixture and the labeling procedure are the same as described above for RNA labeling, except that the Klenow fragment of DNA polymerase I (5 μg/μL, Gibco BRL Life Science Technologies) was used as the enzyme. After incubation 37 ^{0}C for 2 hr, the labeled DNA probe was purified using a PCR purification kit (Qiagen, Valencia, CA).

Hybridization and washing. Slides were first incubated with prehybridization solution containing 3.5xSSC (BRL, Life Technologies Inc., Gaithersburg, MD), 0.1% SDS (BRL, Life Technologies Inc., Gaithersburg, MD), 1% bovine serum albumin (BSA, Fraction V, Sigma, St. Louis, MO). After prehybridization, hybridization solutions (Molecular Dynamics)

containing labeled probes was added to slides and covered with cover slips. Slides were placed in a humidified chamber in a 42°C incubator. After overnight hybridization, slides were initially washed for 5 min at room temperature with a washing solution containing 1xSSC, 0.1 % SDS and 0.1xSSC, 0.1% SDS. Slides were then washed at 65°C for 10 min with the same solution for three times. After washing, the slides were dried with a stream of nitrogen gas.

Data Collection and Analysis. The signal generated from each slide was quantified with a laser scanner (Molecular Dynamics, Sunnyvale, CA). The images were analyzed with ArrayVision 4.0 software (Imaging Research, Inc., Ontario, Canada). The raw fluorescent intensity for each spot was adjusted by subtracting the background. These readings were exported to a spreadsheet for further analysis.

Table 1 is a description of the genes discovered and annotated for *Methylomonas* 16a. The table shows sequence % similarities, % identities, and expectation values for key genes of central carbon metabolism, denitrification, exopolysacharride synthesis, and isoprenoid biosynthesis.

Table 1 illustrates the relationship of these sequences to known sequences in the art. All sequences were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul, S. F., et al., (1993) *J. Mol. Biol.* 215:403-410; see also www.ncbi.nlm.nih.gov/BLAST/) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The sequences were analyzed for similarity to all publicly available DNA sequences contained in the "nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish, W. and States, D. J. (1993) *Nature Genetics 3*:266-272) provided by the NCBI. All comparisons were done using either the BLASTNnr or BLASTXnr algorithm. The results of the BLAST comparison is given below in Table 1 which summarize the sequences to which they have the most similarity. Table 1 displays data based on the BLASTXnr algorithm with values reported in expect values. The Expect value estimates the statistical significance of the match,

specifying the number of matches, with a given score, that are expected in a search of a database of this size absolutely by chance.

Table 1
Genes Characterized From *Methylomonas* 16a

Gene Name	Similarity Identified	SEQ ID	SEQ ID Peptide	% Identity [a]	% Similarity [b]	E-value [c]	Citation
Phosphoglucomutase	Phosphoglucomutase (Glucose Phosphomutase) (Pgm)>>gi|3241933|gb|AAD03475.1|	1	2	65%	85%	1.7e-140	Lepek et al., Direct Submission |gb|AAD03475.1|
Glucose 6 phosphate isomerase	Glucose 6 phosphate isomerase gi|396360|gb|AAC43119.1	3	4	64%	81%	1.6e-136	Blattner et al., Nucleic Acids Res. 21 (23), 5408-5417 (1993)
Phosphofructokinase pyrophosphate dependent	Phosphofructokinase pyrophosphate dependent gi|150931|gb|AAA25675.1| (M67447)	5	6	63%	83%	1.7e-97	Ladror et al., J. Biol. Chem. 266, 16550-16555 (1991)
6-Phosphogluconate dehydratase	6-Phosphogluconate dehydratase gi|4210902|gb|AAD12045.1| (AF045609)	7	8	60%	85%	1.6e-141	Willis et al., J. Bacteriol. 181 (14), 4176-4184 (1999)

Gene Name	Similarity Identified	SEQ ID	SEQ ID Peptide	% Identity [a]	% Similarity [b]	E-value [c]	Citation
Glucose 6 phosphate 1 dehydrogenase	Glucose 6 phosphate 1 dehydrogenase gi|397854|emb|CAA52858.1| (X74866)	9	10	58%	85%	9.4e-123	Hugouvieux-Cotte-Pattat,N, TITLE Direct Submission, gi|397854|emb|CAA52858.1| (X74866)
TAL	Transaldolase	11	12	78%	90%	2.7e-92	Plant Mol. Biol. 30 (1), 213-218 (1996)
MIPB	Transaldolase	13	14	50%	79%	1e-23	Blattner F.R. et. al Science 277:1453-1474(1997).
FBA or FDA	Fructose bisphosphate aldolase	15	16	76%	92%	4.1e-111	Alefounder P.R. et. al. Mol. Microbiol. 3:723-732(1989).
FBA or FDA	Fructose bisphosphate aldolase	17	18	40%	70%	2.3e-39	van den Bergh E.R. et al.; J. Bacteriol. 178:888-893 (1996).
KHG/KDPG	(AL352972) KHG/KDPG aldolase Streptomyces coelicolor	19	20	59%	72%	1e-64	Redenbach et al., Mol. Microbiol. 21 (1), 77-96 (1996)
ugp	*ugp (Xanthomonas campestris)*	21	22	58%	82%	3.2 e-60	Wei *et al.*, *Biochem. Biophys. Res. Commun.* 226 (3), 607-612 (1996)

Gene Name	Similarity Identified	SEQ ID	SEQ ID Peptide	% Identity [a]	% Similarity [b]	E-value [c]	Citation
gumD	*gumD (Xanthomonas campestris)*	23	24	36%	69%	2.5 e-52	Chou,F.L., *et el, Biochem. Biophys. Res. Commun.* 233 (1), 265-269 (1997)
wza	wza (Escherichia coli)	25	26	36%	69%	5.8 e-39	Blattner,F.R.et al., Science 277 (5331), 1453-1474 (1997)
epsB	*epsB (Pseudomonas solanacearum)*	27	28	35%	67%	2 e-74	Huang,J. and Schell,M., *Mol. Microbiol.* 16 (5), 977-989 (1995)
epsM	*epsM (Streptococcus thermophilus)*	30	20	23%	55%	1.3 e-05	Stingele,F. et al., , *J. Bacteriol.* 178 (6), 1680-1690 (1996)
waaE	*waaE (Serratia marcescens)*	31	32	28%	55%	8.6 e-09	Pique,N et al., . Unpublished Genbank number: AAC44433
epsV	*epsV (Streptococcus thermophilus)*	33	34	21%	56%	2.3 e-05	Bourgoin,F. et al., Plasmid 40 (1), 44-49 (1998)
gumH	*gumH (Rhizobium meliloti)*	35	36	26%	55%	0.00088	Becker,A. et al., Mol. Microbiol. 16 (2), 191-203 (1995)

Gene Name	Similarity Identified	SEQ ID	SEQ ID Peptide	% Identity [a]	% Similarity [b]	E-value [c]	Citation
glycosyl transferase	Glycosyltransferase (*Actinobacillus actinimycetemcomitans*)	37	38	51%	80%	1.7 e-62	Nakano,Y, Biochem. Biophys. Acta 1442:409-414 (1998)
nirF	NirF protein (*Pseudomonas*)	39	40	59%	85%	1.3e-92	Palmedo et al., Eur. J. Biochem. 232 (3), 737-746 (1995)
nirD	NirD protein (*Pseudomonas*)	41	42	49%	76%	1.7e-22	Palmedo et al., Eur. J. Biochem. 232 (3), 737-746 (1995)
nirL	NirL protein (*Pseudomonas*)	43	44	49%	73%	6.4e-28	Palmedo et al., Eur. J. Biochem. 232 (3), 737-746 (1995)
nirG	NirG protein (*Pseudomonas*)	45	46	49%	80%	1.6e-25	Kawasaki et al., J. Bacteriol. 179 (1), 235-242 (1997)
nirH	NirH protein (*Pseudomonas*)	47	48	59%	78%	9.9e-33	Kawasaki et al., J. Bacteriol. 179 (1), 235-242 (1997)
nirJ	NirJ protein (*Pseudomonas*)	49	50	56%	81%	5.1e-88	Kawasaki et al., J. Bacteriol. 179 (1), 235-242 (1997)
nasA	Nitrate reductase *Klebsiella*	51	52	51%	74%	9.2e-123	LIN J.T., GOLDMAN B.S., STEWART V.; J. Bacteriol. 175:2370-2378(1993).

Gene Name	Similarity Identified	SEQ ID	SEQ ID Peptide	% Identity [a]	% Similarity [b]	E-value [c]	Citation
norC	Nitric-oxide reductase subunit C (*Pseudomonas*)	53	54	32%	70%	1e-08	Zumft et al., Eur. J. Biochem. 219:481-490(1994).
norB	Nitric-oxide reductase subunit B (*Pseudomonas*)	55	56	39%	70%	3.5e-64	Zumft et al., Eur. J. Biochem. 219:481-490(1994).
norZ	Cytochrome B subunit of nitric oxide reductase (*Alcaligenes*)	57	58	39%	69%	1.7e-100	Cramm,R., Siddiqui,R.A. and Friedrich,B. J. Bacteriol. 179 (21), 6769-6777 (1997).
norS	Nitrite reductase (cytochrome cd1) (*Pseudomonas*)	59	60	28%	59%	2.1e-25	Glockner,A.B. and Zumft,W.G. Biochim. Biophys. Acta 1277 (1-2), 6-12 (1996)
dxs	1-deoxyxylulose-5-phosphate synthase	61	62	60%	86%	5.7e-149	Lois,L.M., et al., Proc. Natl. Acad. Sci. U.S.A. 95 (5), 2105-2110 (1998)

Gene Name	Similarity Identified	SEQ ID	SEQ ID Peptide	% Identity [a]	% Similarity [b]	E-value [c]	Citation
dxr	1-deoxy-d-xylulose 5-phosphate reductoisomerase	63	64	55%	78%	3.3e-74	Takahashi S et al., Proc. Natl. Acad. Sci. U.S.A. 95:9879-9884(1998).
ygbB/ispF	2C-methyl-d-erythritol 2,4-cyclodiphosphate synthase	65	66	69%	84%	1.6e-36	Herz S, et al., *Proc Natl Acad Sci U S A* 2000 Mar 14;97(6):2486-90
ygbP/ispD	2C-methyl-d-erythritol cytidylyltransferase	67	68	52%	74%	7.7e-36	Rohdich F, et al., *Proc Natl Acad Sci U S A* 1999 Oct 12;96(21):11758-63
pyrG	CTP synthase	69	70	67%	89%	2.4e-141	Weng M., J. et al., Biol. Chem. 261:5568-5574(1986).
lspA	Geranyltransferase (also farnesyl-diphosphate synthase)	71	72	56%	78%	7.8e-56	Ohto,C et al., Plant Mol. Biol. 40 (2), 307-321 (1999)
ychB/lspE	4-diphosphocytidyl-2-C-methylerythritol kinase	73	74	50%	72%	8.8e-49	Luttgen H, Proc Natl Acad Sci U S A. 2000 Feb 1;97(3):1062-7.

Gene Name	Similarity Identified	SEQ ID	SEQ ID Peptide	% Identity [a]	% Similarity [b]	E-value [c]	Citation
crtN1	diapophytoene dehydrogenase CrtN—copy 1	75	76	34%	72%	4e-66	Xiong,J Proc. Natl. Acad. Sci. U.S.A. 95 (25), 14851-14856 (1998)
crtN2	Diapophytoene dehydrogenase CrtN—copy 2	77	78	49%	78%	1.3e-76	Wieland,K.P. and Goetz,F. Unpublished
Particulate methane monooxygenase	probable methane monooxygenase 45k chain - Methylococcus capsulatus B57266 GI:2120829	79	80	71%	85%	0.0	Semrau et al., J. Bacteriol. 177 (11), 3071-3079 (1995)

[a]%Identity is defined as percentage of amino acids that are identical between the two proteins.

[b]% Similarity is defined as percentage of amino acids that are identical or conserved between the two proteins.

[c]Expect value. The Expect value estimates the statistical significance of the match, specifying the number of matches, with a given score, that are expected in a search of a database of this size absolutely by chance

EXAMPLE 1

ISOLATION OF *METHYLOMONAS* 16A

The original environmental sample containing the isolate was obtained from pond sediment. The pond sediment was inoculated directly into defined medium with ammonium as nitrogen source under 25% methane in air. Methane was the sole source of carbon and energy. Growth was followed until the optical density at 660 nm was stable whereupon the culture was transferred to fresh medium such that a 1:100 dilution was achieved. After 3 successive transfers with methane as sole carbon and energy source the culture was plated onto growth agar with ammonium as nitrogen source and incubated under 25% methane in air. Many methanotrophic bacterial species were isolated in this manner. However, *Methylomonas* 16a was selected as the organism to study due to the rapid growth of colonies, large colony size, ability to grow on minimal media, and pink pigmentation indicative of an active biosynthetic pathway for carotenoids.

EXAMPLE 2

RAPID GROWTH ON METHANE IN MINIMAL MEDIUM

Methylomonas 16a grows on the defined medium comprised of only minimal salts, a culture headspace comprised of methane in air. Methane concentrations for growth but typically are 5-50% by volume of the culture headspace. No organic additions such as yeast extract or vitamins are required to achieve growth shown in Figure 1. Figure 1 shows the growth of 16a compared to the growth of *Methylococcus capsulatus* under identical growth conditions. i.e. minimal medium with 25% methane in air as substrate. The data indicates *Methylomonas* 16a doubles every 2-2.5 h whereas *Methylococcus capsulatus* doubles every 3.5 h with methane as substrate. With methanol as substrate doubling times on methanol are 2.5-3 for *Methylomonas* 16a and 4.5-5 for *Methylococcus capsulatus*. Cell densities are also significantly higher for *Methylomonas* 16a growing on methane. *Methylococcus capsulatus* is a widely utilized methanotroph for experimental and commercial purposes.

EXAMPLE 3

METHANOL TOLERANCE

Methylomonas 16a was grown on defined medium with nitrate as sole nitrogen source and methanol as sole carbon source. Growth was monitored over a 36 hr period which was typically sufficient for attaining maximum optical density or turbidity of the culture. Figure 2 clearly shows

that maximum growth or turbidity is attained within 36 hours at methanol concentrations up to 600 mM. However no growth was observed at 800 mM. Therefore the strain is shown to grow on 2.4% (vol/vol) of methanol.

EXAMPLE 4

PROPERTIES AND CLASSIFICATION OF *METHYLOMONAS 16A*

Table 2 shows the various properties of *Methylomonas* 16a. The criteria listed in Table 2 are those typically used to determine whether the strain is arbitrarily considered Type I, Type II or Type X based on physical and enzymatic properties. This table was developed from both direct enzymatic assay for enzymes as well as genomic data showing the presence of genes and gene pathways. This categorization is functionally based and indicates that the strain utilizes the most energetically efficient pathway for carbon incorporation which is the ribulose monophosphate or "RuMP" pathway. Genomic data clearly shows the presence of key enzymes in the RuMP pathway. Internal membrane structure are also indicative of a Type I physiology. Unique to the present strain is the finding of nitrogen fixation genes in *Methylomonas* 16a. The strain is shown to grow in the absence of yeast extract or vitamins. Nitrate, ammonium ion or dinitrogen can satisfy the nitrogen requirement for biosynthesis. This functional data is in complete agreement with the 16srRNA homologies as compared with other *Methylomonas* strains. 16sRNA comparisons or the 16a strain (SEQ ID NO:81) with other *Methylomonas sp.* revealed that *Methylomonas* 16a has 96% identity with the 16sRNA of *Methylomonas sp.* (strain:KSPIII) [Hanada,S et al., *J. Ferment. Bioeng.* 86, 539-544 (1998)] and with *Methylomonas* sp. (strain LW13), [Costello, A.M. and Lidstrom, M.E. *Appl. Environ. Microbiol.* 65 (11), 5066-5074 (1999)]]. Thus *Methylomonas* 16a is correctly classified as a Type I, RuMP utilizing, *Methylomonas* species.

Table 2

Characteristic	Type I	*Methylomonas* 16a	Type X	Type II
%GC	Incomplete	Incomplete	Incomplete	Complete
Ribmp Cycle	Incomplete	Incomplete	Incomplete	Complete
RuBP Carboxylase	-	-	+	+
Temp. Range	<45	<42	<45	<40
Nitrogenase	-	+	+	+
G6P dehydrogenase NADP	+	+	+	-
Isocitrate dehydrogenase NAD/NADP	+	+	-	-
Yeast Extract	-	-	-	-
Vitamins	-	-	-	-
Pigmentation	Variable	+	Variable	Variable
Nitrate assimilation	+	+	+	+

Method of enzymatic assay

Nitrogenase was not assayed but is considered positive if the gene is present on the basis of genome sequence analysis.

Glucose 6 phosphate dehydrogenase: One mL of reaction mixture contains 100 µL of 10 mM NADP, 100 µL of 10 mM glucose, 700 µL of 100 mM HEPES pH 7 buffer and up to 100 µL of enzyme extract. The enzyme activity was measured by monitoring NADP reduction to NADPH at 340 nm using spectrophotometer.

Isocitrate dehydrogenase: One mL of reaction mixture contains 100 µL of 10 mM sodium isocitrate, 100 µL of 10 mM NADP, 700 µL of 100 mM pH 7 HEPES buffer up to 100 µL of enzyme extract. The enzyme activity was measured by monitoring NADPH formation at 340 nm.

Nitrate assimilation is based on the ability of the strain to grow on nitrate as sole nitrogen source.

The results of the enzyme assay are sown in Table 2.

EXAMPLE 5

COMPARISON OF GENE EXPRESSION LEVELS IN THE ENTNER DOUDEROFF PATHWAY AS COMPARED WITH THE EMBEDEN MEYERHOF PATHWAY

Example 5 presents microarray evidence for the use of the Embden-Meyerhof pathway in the 16a strain.

Figure 3 shows the relative levels of expression of genes for the Entner-Douderoff pathway and the Embden-Meyerhof pathway. The relative transcriptional activity of each gene was estimated with DNA microarray as described previously (Wei, *et al.*, 2001. *Journal of Bacteriology*. 183:545-556).

Specifically, a single DNA microarray containing 4000 ORFs (open reading frames) of *Methylomonas sp.* strain 16a was hybridized with probes generated from genomic DNA and total RNA. The genomic DNA of 16a was labeled with Klenow fragment of DNA polymerase and fluorescent dye Cy-5, while the total RNA was labeled with reverse transcriptase and Cy-3. After hybridization, the signal intensities of both Cy-3 and Cy-5 for each spot in the array were quantified. The intensity ratio of Cy-3 and Cy-5 was then used to calculate the fraction of each transcript (in percentage) with the following formula: (gene ratio/sum of all ratio) x 100. The value obtained reflects the relative abundance of mRNA of an individual gene. Accordingly, transcriptional activity of all the genes represented by the array can be ranked based on its relative mRNA abundance in a descending order. For example, mRNA abundance for the methane monooxygenase was ranked #1 because its genes had the highest transcriptional activity when the organism was grown with methane as the carbon source (Figure 3).

The genes considered "diagnostic" for Entner-Douderoff are the 6 phosphogluconate dehydratase and the 2 keto-3-deoxy-6-phosphogluconate aldolase. Phosphofructokinase and fructose bisphosphate aldolase are "diagnostic" of the Embden-Meyerhof sequence. Numbers in Figure 3 next to each step indicate the relative expression level of that enzyme. For example the most highly expressed enzyme in the cell is the methane monooxygenase (ranked #1). The next most highly expressed is the methanol dehydrogenase (ranked #2). Messenger RNA transcripts of Phosphofructokinase (ranked #232) and fructose bisphosphate aldolase (ranked #65) were in higher abundance than those for glucose 6 phosphate dehydrogenase (ranked #717), 6

phosphogluconate dehydratase (ranked #763) or the 2-keto-3-deoxy-6-gluconate aldolase. The data suggests that the Embden-Meyerhof pathway enzymes are more strongly expressed than the Entner-Douderoff pathway enzymes. This result is surprising and counter to existing beliefs on the central metabolism of methanotrophic bacteria (Reference book pages in. The physiology and biochemistry of aerobic methanol-utilizing gram-negative and gram- positive bacteria In: Methane and Methanol Utilizers, Biotechnology Handbooks 5. 1992. Eds: Colin Murrell, Howard Dalton. Pp 149-157.

EXAMPLE 6

DIRECT ENZYMATIC EVIDENCE FOR A PYROPHOSPHATE-LINKED PHOSPHOFRUCTOKINASE

Example 6 shows the evidence for the presence of a pyrophosphate-linked phosphofructokinase enzyme in the current strain which would confirm the functionality of the Embden-Meyerhof pathway in the present strain.

Phosphofructokinase activity was shown to be present in *Methylomonas* 16a by using the coupled enzyme assay described below. Assay conditions are given in Table 3 below. This assay was further used to assay the activity in a number of other Methanotrophic bacteria as shown below in Table 4. The data in Table 4 show known ATCC strains tested for phosphofructokinase activity with ATP or pyrophosphate as phosphoryl donor. These organisms were classified as either Type I or Type X ribulose monophosphate-utilizing strains or Type II serine utilizer.

Coupled Assay Reactions

Phosphofructokinase reaction is measured by a coupled enzyme assay. Phosphofructokinase reaction is coupled with fructose 1,6, biphosphate aldolase followed by triosephosphate isomerase. The enzyme activity is measured by the disappearance of NADH.

Specifically, the enzyme phosphofructokinase catalyzes the key reaction converting Fructose 6 phosphate and pyrophosphate to Fructose 1,6 bisphosphate and orthophosphate.

Fructose-1,6-bisphosphate is cleaved to 3-phosphoglyceraldehyde and dihydroxyacetonephosphate by fructose 1,6-bisphosphate aldolase.

Dihydroxyacetonephosphate is isomerized to 3-phosphoglyceraldehyde by triosephosphate isomerase.

Glycerol phosphate dehydrogenase plus NADH and 3-phosphoglyceraldehyde yields the alcohol glycerol-3-phosphate and NAD.

Disappearance of NADH is monitored at 340 nm using spectrophotometer (UltraSpec 4000, Pharmacia Biotech).

Table 3

Assay Protocol

Reagent	Stock solution (mM)	Volume (µl) per 1mL total reaction volume	Final assay concentration (mM)
Tris-HCl pH 7.5	1000	100	100
$MgCl_2$. 2 H_2O	100	35	3.5
$Na_4P_2O_7.10H_2O$ or ATP	100	20	2
Fructose-6-phophate	100	20	2
NADH	50	6	0.3
Fructose bisphosphate aldolase	100 (units/mL)	20	2 (units)
Triose phosphate isomerase/glycerol phosphate dehydrogenase	(7.2 units/µl) (0.5 units/µl)	3.69	27 units 1.8 units
KCl	1000	50	50
H2O		adjust to 1mL	
Crude extract		0-50	

Table 4

Comparison Of Pyrophosphate Linked And ATP Linked Phosphofructokinase Activity In Different Methanotrophic Bacteria

Strain	Type	Assimilation Pathway	ATP-PFK umol NADH/ min/mg	Ppi-PFK umol NADH/ min/mg
Methylomonas 16a ATCC PTA 2402	I	Ribulose monophosphate	0	2.8
Methylomonas agile ATCC 35068	I	Ribulose monophosphate	0.01	3.5
Methylobacter Whittenbury ATCC 51738	I	Ribulose monophosphate	0.01	0.025
Methylomonas clara ATCC 31226	I	Ribulose monophosphate	0	0.3
Methylomicrobium albus ATCC 33003	I	Ribulose monophosphate	0.02	3.6
Methylococcus capsulatus ATCC 19069	X	Ribulose monophosphate	0.01	0.04
Methylosinus sporium ATCC 35069	II	Serine	0.07	0.4

Several conclusions may be drawn from the data presented above. First, it is clear that ATP (which is the typical phosphoryl donor for phosphofructokinase) is essentially ineffective in the phosphofructokinase reaction in methanotrophic bacteria. Only inorganic pyrophosphate was found to support the reaction in all methanotrophs tested. Secondly not all methanotrophs contain this activity. The activity was essentially absent in *Methylobacter whittenbury* and *in Methylococcus capsulatus*. Intermediate levels of activity were found in *Methylomonas clara* and *Methylosinus sporium*. These data show that many methanotrophic bacteria may contain a hitherto unreported phosphofructokinase activity. It may be inferred from this that methanotrophs containing this activity have an active Embden-Meyerhof pathway.

EXAMPLE 7

GROWTH YIELD AND CARBON CONVERSION BY METHYLOMONAS 16A

Growth yield and carbon conversion efficiency were compared for *Methylomonas 16a* and *Methylococcus capsulatus*. These strains were chosen because 16a contains high levels of phosphofructokinase and *M. capsulatus* is essentially devoid of the enzyme activity. It was contemplated that if *Methylomonas* 16a could utilize the more energetically favorable Embden-Meyerhof pathway and *Methylococcus capsulatus* could only use the Entner-Douderoff pathway the superior energetics of the present *Methylomonas 16a* strain would be reflected in cellular yields and carbon conversion efficiency. This difference in energetic efficiency would only be apparent under energy-limiting conditions. These conditions were achieved in this experiment by limiting the amount of oxygen in each culture to only 10% (vol/vol) instead of 20% (growth conditions employed in Figure 1 and Table 9). Under these oxygen limiting conditions the strain that produces the most energy from aerobic respiration on methane will produce more cell mass.

Cells were grown as 200 mL cultures 500 mL serum-stoppered Wheaton bottles. The headspace in the bottles was adjusted to 25% methane and 10% oxygen. The defined medium formulation is the same in both cases.

Table 5

Yield Of Methylomonas 16a Cells Versus Methylococcus Capsulatus Cells Under Oxygen Limitation.

Strain	Y_{CH4} g dry wt/mol	G dry wt/g CH_4	Carbon Conversion Efficiency (CCE)%
Methylomonas 16a	16.7 +/- 0.5	1.04	64
Methylococcus capsulatus	10.3 +/- 0.3	0.64	40

Yield determination: Yield was measured by growing triplicate cultures in 500 mL bottles on defined medium with ammonium as nitrogen source under oxygen limitation. This was done by using 300 mL of culture with a 300 mL headspace of 25% methane and 10% oxygen the balance

being nitrogen. At the end of growth (i.e. stationary phase) residual methane in the headspace was determined by gas chromatography. The cells were collected by centrifugation washed with distilled water and dried overnight in a drying oven before being weighed.

Carbon conversion efficiency is a measure of how much carbon is assimilated into cell mass. It is calculated assuming a biomass composition of $CH_2O_{0.5}N_{0.25}$:

Methylomonas 16a : 16 g/mol methane X (1 g dry wt/g methane) / 25 g/ mol biomass

M. capsulatus 16 g/mol methane X (0.64 g dry wt/g methane) / 25 g /mol biomass

These data (in Table 5) show that *Methylomonas 16a* produced significantly more cell mass than did the *Methylococcus capsulatus* strain under growth conditions that were identical except for the temperature. *Methylococcus capsulatus* grows optimally at 45°C whereas Methylomonas is grown at 33°C. It may be inferred from the data that the presence of the more energy-yielding Embden-Meyerhof pathway confers a growth advantage to *Methylomonas 16a*.

Table 6 presents the theoretical calculations showing ATP yield as a function of carbon assimilation pathway with the carbon output being normalized to pyruvate in all cases (The physiology and biochemistry of aerobic methanol-utilizing gram-negative and gram- positive bacteria In: Methane and Methanol Utilizers, Biotechnology Handbooks 5. 1992. Eds: Colin Murrell, Howard Dalton. Pp. 149-157). Table 6 shows the amount of ATP that is produced or consumed for every three molecules of carbon (as formaldehyde or carbon dioxide) for serine cycle, xylulose monophosphate cycle and ribulose monophosphate cycle pathways. The latter pathway, as discussed is typically thought to exist as the 2-keto-3deoxy-6-phosphogluconate /transaldolase (KDPGA/TA) variant. These data shows that in fact the fructose bisphosphate aldolase/transaldolase (FBPA/TA) variant is likely to exist in the methanotrophs. The energetic repercussion of this is the net production of an additional 1 ATP for methanotrophs if they possess an ATP linked phosphofructokinase and an additional 2 ATPs for the pyrophosphate-linked enzyme. It is therefore expected that *Methylomonas 16a* derives and additional 2 ATP per 3 carbons assimilated and that this may explain the greater yield and carbon efficiency of the strain versus *Methylococcus capsulatus*.

Table 6

Energetics of Methanotrophic bacteria utilizing different carbon assimilation mechanisms

Organism	Cycle	C1 unit fixed	Product	Variant	ATP	NADPH
Bacteria	RuMP	$3CH_2O$	Pyruvate	FBPA/TA	+1	+1
Methylomonas	RuMP/Serine	$3CH_2O$	Pyruvate	FBPA/TA	+1(+2*)	+1
Bacteria	RuMP	$3CH_2O$	Pyruvate	KDPGA/TA	0	+1
Methylococcus	RuMP/RuBP	$3CH_2O$	Pyruvate	KDPGA/TA	0	+1

* Based on PPi dependent phosphofructokinase

EXAMPLE 8

NITRATE/NITRITE SPARES OXYGEN

Figure 4 shows oxygen uptake by a cell suspension of *Methylomonas* 16a, in relative detector units, using an Orion oxygen probe (Orion, UK) to detect oxygen consumption. Oxygen was measured as a function of time in the presence or absence of nitrate and in the presence of methanol as electron donor and carbon source. The incubation consisted of *Methylomonas* 16a cells suspended in HEPES buffer pH 7. Methanol was injected at 3 min into both incubations to achieve a final concentration of about 100 mM. After the methanol injection it can be seen that oxygen uptake accelerated as would be expected (Figure 4) in the cultures without nitrate. However the rate of oxygen uptake in the presence of nitrate never approaches that of cells without nitrate. The data thus supports the finding that nitrate can spare oxygen consumption with methanol as carbon source.

Methylomonas 16a cells were again suspended in HEPES buffer pH 7 and incubated in a water jacketed chamber equipped with an Orion oxygen probe. The incubation was carried out at 30°C. Methanol was injected into the incubation at 1 min. However in one incubation sodium nitrite (25 mM) was injected into the incubation after 23 min. The results are shown in Figure 5. As seen in Figure 5, there is a decrease in the rate of oxygen uptake after the addition of nitrite. This data again clearly supports the assertion that nitrite and indirectly nitrate can be used as an alternative electron sink and resulting in less oxygen consumption by the culture.

A cell suspension of *Methylomonas* 16a in defined medium under 25% methane in air was simultaneously monitored for oxygen and N_2O in the dead-space. 100 mM Nitrite was the only added source of nitrogen. The results are shown in Figure 6. Figure 6 illustrates that the appearance of N_2O in the dead-space coincides with oxygen depletion. The numbers plotted are the rates of appearance or disappearance of N_2O and oxygen respectively. As oxygen disappearance rates decline to lower values (due to lower headspace O_2 concentrations) N_2O production increases to become a significant fraction of the total electron flow through the organism (only under oxygen limitation).

EXAMPLE 9

NITRATE OR NITRITE REDUCTION BY OTHER STRAINS OF METHANOTROPHS AND *METHYLOMONAS* 16A.

All methanotrophic strains available from the American Type Culture collection were tested for their ability to produce N_2O from nitrite or nitrate. All strains were grown on the defined medium and harvested after an optical density at 660 nm of 1.0 was achieved. The cell suspensions were collected by centrifugation and resuspended in 5 mL of defined medium with either nitrate or nitrite as sole nitrogen source. The data in Table 7 below shows the accumulation of N_2O (in uM concentration) in the headspace of a 10 mL assay vial incubated 30°C. The results shows that *Methylomonas* 16a has a unique ability to convert nitrate to N_2O among the strains tested. Furthermore the data show that two other *Methylomonas* strains have a similar ability to convert nitrite to N_2O.

Table 7

STRAIN	NO_3/NO_2 uM	NO_2/N_2O uM
Methylomonas 16a	28.3	30
Methylomonas albus	1.2	22
Methylomonas clara	2.5	1.5
Methylomonas agile	0.6	17
Methylobacter whitterbury	0.3	0.04
Methylococcus capsulatis	0.3	1.9
Methylobacter lutes	0.1	6.5
Methylosinus sporium	0.2	0.07

EXAMPLE 10

PRODUCTION OF GLYCOGEN.

Methylomonas 16a was shown to accumulate large amounts of glycogen when grown on either methane or methanol. *Methylomonas* cells were analyzed for glycogen using a starch assay kit (Sigma Chemical Co. St Louis MO). This assay is starch or glycogen specific and conclusively shows the presence of glycogen in *Methylomonas* 16a. Cells were grown according to the conditions outlined in the General Methods, Cells were harvested during growth on 100 mM methanol or 25% headspace methane at 30°C on defined medium. Culture samples were taken at two points in the growth curve: mid-logarithmic growth (O.D. 660 0.3) and stationary phase (O.D. 660 1.0). These samples were immediately analyzed with the starch assay kit according to the manufacturers instructions. The results shown below in Table 8 indicate surprising amounts of the storage polymer during growth on methanol and lower but significant amounts of glycogen during growth on methane.

Table 8

Growth Phase (OD660)	Methane (%glycogen wt/wt)	Methanol (% glycogen (wt/wt)
Mid-log (0.3)	6%	25%
Stationary phase (1.0)	7%	40%

Additionally, the presence of granules within the cells grown on methanol were observed by scanning electron microscopy and the granules were determined to contain starch with polysaccharide specific stains.

EXAMPLE 11

PRODUCTION OF PROTEIN FROM CELL MASS

Methylomonas 16a and *Methylococcus capsulatus* (reference strain for protein production) were grown on defined medium until no further increases in OD 660 could be observed. Methane or methanol consumption was monitored by gas chromatography (HP-Plot Molecular sieve column; Hewlett Packard 5890 series II gas chromatograph) over the growth curve such that the total amount of methane or methanol consumed could be calculated. The running conditions for GC were; oven

temperature: 40°C, initial temperature: 40°C, initial time: 3 min, rate: 0 deg/min, final temperature 40°C, final time 0, injection A temperature: 100°C, Det. A temperature: 125°C, and equilibration time: 0.

The cells were collected by centrifugation and dried overnight in a 105°C drying oven. The data in Table 9 below shows the gram dry weight of cells produced per gram of methane or methanol consumed.

Table 9

Organism	g dry wt./g CH_4	g dry wt./g CH_4OH
Methylomonas 16a	0.90 - 1.3 (2-2.5 hr)	0.30 - 0.45 (2.5 - 3.0 hr)
Methylococcus capsulatus	0.67 - 1.2 (3 - 4 hr)	0.25 - 0.45 (4 -5 hr)

As can be seen by the data in Table 9 the present strain has a higher rate of protein production than the commercial methanotroph of choice for this process, when grown on either methane or methanol.

EXAMPLE 12

PRODUCTION OF EXTRACELLULAR POLYSACHARRIDE

Methylomonas 16a cells were grown on 25% methane in 200 mL batch culture on defined medium at 30°C. Initial oxygen concentration was varied by injecting pure oxygen into nitrogen flushed bottles. Cells were allowed to grow until stationary phase or to an optical density of approximately 1.0. At that time the cultures were centrifuged at 6000 x g for 30 min to sediment both the cells and the extracellular polysaccharide. The sediments from these centrifugations comprised two layers. At the bottom were the cells, overlaid with a clear viscous material which was the extracellular polysaccharide (EPS). The EPS layer was washed off and pelleted again for further separation from the cells. The cell pellet was also dried and weighed. The EPS was resuspended in 50% ethanol and pelleted again in the centrifuge. Finally the material was dried and weighed. EPS was found to comprise as much as 50% of the total dry weight of the culture at near-ambient oxygen concentrations. This was determined by centrifugation of the culture at 10,000 x g for 30 min. The resulting pellet is comprised of a lower red phase (packed cells) and an upper translucent phase which is the extracellular polysaccharide. The EPS was selectively removed with a spatula and dried at 105°C overnight. The cell pellet was removed and dried at 105°C overnight. The

supernatant from the centrifugation was mixed with cold isopropanol (1:1 vol:vol). The precipitated EPS from this step was collected by centrifugation (10,000 x g for 30 min) and the pellet dried at 105°C overnight and weighed. Chemical analysis of the EPS revealed that it was primarily polyglucose (~70%). EPS samples were methylated by the method of Ciucanu, I., F. Kerek. 1984. *Carbohydrate Research* 131:209-217. The methylated samples were hydrolyzed in 2 M TFA at 121°C for 2 hours and the hydrolyzed carbohydrate was reduced with sodium borodeuteride at room temperature. The product was acetylated by GC-MS using Sp2330 Supelco column. Internal standard myo-inositol was added to each sample prior to the reduction step.